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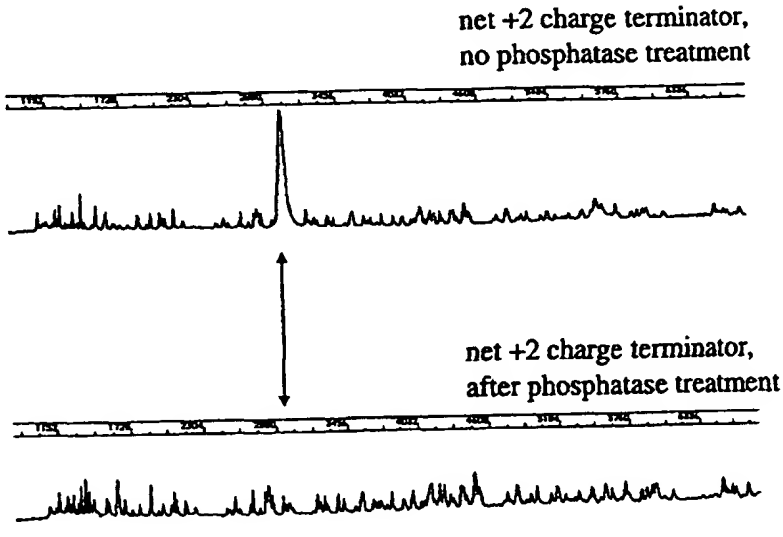
(54) Title: CHARGE-MODIFIED NUCLEIC ACID TERMINATORS



(57) Abstract: Charge-modified nucleic acid terminators comprising structure (I): Z-X-S-B-L wherein Z is mono-, di- or triphosphate or thiophosphate, or corresponding boranophosphate; X is O, CH_2, S, or NH; S is a sugar or a sugar analogue; B is a naturally occuring or a synthetic base; L is alkyl, alkenyl, or alkynyl and is optionally substituted with a reporter moiety; and L, B, S, X, or Z are substituted with a moiety which imparts a net negative charge or a net positive charge to structure (I) at physiological or nucleic acid sequencing conditions. A method of sequencing nucleic acids using the above charge-modified terminators, as well as a method of inhibiting a virus which comprises contacting a cell infected with a virus with a virus-inhibiting amount of the above charge-modified terminator are also disclosed.

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CHARGE MODIFIED NUCLEIC ACID TERMINATORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to United States Provisional Application Serial No. 60/154,739, filed on September 17, 1999, the entire disclosure of which is incorporated in its entirety herein.

BACKGROUND OF THE INVENTION

Field of the Invention

The instant invention pertains to nucleic acid terminators which result in improved sequence data. The instant invention also pertains to charge modified nucleic acid terminators which allow for the direct loading of nucleic acid sequencing reactions onto separating media.

BACKGROUND OF THE INVENTION

The sequence of nucleotide bases in a DNA molecule can be determined in a variety of ways. The chain termination method generally involves synthesizing DNA complementary to the template strand to be sequenced by extending a primer able to hybridize to a portion of that template strand with a DNA polymerase. During the synthesis reaction, deoxynucleoside triphosphates (dNTP's) are incorporated to form a DNA fragment until a chain terminating agent, for example, a dideoxynucleotide triphosphate (ddNTP) is incorporated. Incorporation of a ddNTP prevents further DNA synthesis (a process called chain termination). The size of each DNA fragment synthesized in this procedure is then determined by gel electrophoresis and this information used to determine the sequence of nucleotides in the original template DNA. For example, Tabor and Richardson, U.S. Patent No. 4,795,699, the entire disclosure of which is incorporated herein, describe a two step sequencing method in which an unlabeled primer is labeled in a labeling step, and then extended in the presence of excess dNTPs and a ddNTP in a chain termination step. In the labeling step, a low concentration of dNTPs is provided (one being labeled) to allow a small amount of primer extension.

In the dideoxy sequencing method, the primer may be labeled, for example with P^{32}, by a process using a polynucleotide kinase. Such labeling allows detection of extended

primers after gel electrophoresis by autoradiography of the resulting gel. Alternatively, a labeled dNTP may be incorporated during the process of DNA synthesis, and the presence of such labeled dNTPs detected by autoradiography or other means. To this end, the dNTP may be labeled either radioactively with P^{32} or S^{35}. In another procedure, the primer can be labeled with one or more fluorescent moieties for detection by fluorescence. In yet another procedure, the ddNTP may be labeled, for example, with a fluorescent marker.

In a sequencing reaction, the terminators partially decompose, most likely due to the thermocycling conditions, and generate labeled by-products which migrate in the separating media, thus interfering with interpretation of the true sequencing fragments. For example, terminator decomposition products and unreacted terminators may appear on sequencing gels or electropherograms as peaks or blobs (e.g., Figure 1, lanes 3 and 4 which show the blobs which result when sequencing reaction products containing conventional terminators are directly loaded onto an electrophoretic gel). At the present time, this problem is addressed by precipitation of the sequencing products using e.g., ethanol precipitation (e.g., Figure 1, lanes 1 and 2 which show gels which result when sequencing reactions containing conventional terminators are subjected to ethanol precipitation prior to being loaded onto an electrophoretic gel). While this reduces the contamination somewhat, the procedure is time consuming and creates a bottle-neck for any high through-put sequencing applications.

Thus, a process is needed for improving the clarity of sequencing data. Such a process would not require additional sample preparation steps. Ideally, such a process would reduce sample preparation time and result in improved sequencing through-put. Moreover, such a method would also be economical to use. These and other concerns are addressed in greater detail below.

SUMMARY OF THE INVENTION

One aspect of the instant disclosure pertains to a charge-modified nucleic acid terminator comprising structure (I)

Z—X—S—B—L (I)

wherein

Z is mono-, di or triphosphate or thiophosphate, or corresponding boranophosphate

X is O, CH_2, S, or NH;

S is a sugar or a sugar analogue;

B is a naturally occurring or a synthetic base;

L is alkyl, alkenyl, or alkynyl and is optionally substituted with a reporter moiety; and

L, B, S, X, or Z are substituted with a moiety which imparts a net negative charge or a net positive charge to structure (I) at physiological or nucleic acid sequencing conditions.

In another aspect, the instant disclosure pertains to methods of sequencing nucleic acids using the above charge-modified terminators. In yet another aspect, the instant disclosure pertains to methods of inhibiting a virus which comprises containing a cell infected with a virus with a virus-inhibiting amount of the above charge-modified terminator.

DETAILED DESCRIPTION

The instant invention pertains to nucleic acid terminators, which along with the corresponding nucleic acid terminator decomposition products, migrate on separation media at different rates than the sequencing reaction products and which result in improved sequence data. Such nucleic acid terminators also allow for the direct loading of nucleic acid sequencing reactions onto separating media. To achieve this goal, negatively or positively charged moieties are attached to the terminator molecule. The unreacted or degraded terminators containing such charged moieties move faster (negatively charged) or in the reverse direction (positively charged) than the DNA sequencing products.

One embodiment of the charge-modified terminator according to the instant disclosure is depicted in structure (I)

Z—X—S—B—L (I)

wherein

Z is mono-, di or triphosphate or thiophosphate, or corresponding boranophosphate

X is O, CH_2, S, or NH;

S is a sugar or a sugar analogue;

B is a naturally occurring or a synthetic base;

L is alkyl, alkenyl, or alkynyl and is optionally substituted with a reporter moiety; and

L, B, S, X, or Z are substituted with a moiety which imparts a net negative charge or a net positive charge to structure (I) at physiological or nucleic acid sequencing conditions.

The base may be any naturally occurring or synthetic base such as A, T, G, or C or analogs thereof, such as 7-deazapurine, inosine, universal bases, etc. Suitable base analogs include those disclosed in WO 99/06422 and WO 97/28177.

The sugar may be furanose, hexose, mono-di-triphosphates, morpholine, didehydro, dideoxyribose, deoxyribose, dioxalone, oxathialane, and analogs thereof.

The linker may be alkyl, alkenyl, or alkynyl and may contain 1 to about 100 atoms and may contains atoms such as C, H, N, O, S and halogen. In general, the linker contains about 2 to about 50 atoms. Preferably, when the terminator molecule contains a net positive charge, the linker contains about 2 to about 25 atoms, more preferably, the linker contains about 11 to about 25 atoms. Preferably, when the terminator molecule contains a net negative charge, the linker contains about 11 to 25 atoms, more preferably, the linker contains about 18 to about 25 atoms.

The linker may optionally be substituted with a label, (also referred to as a "reporter or signal moiety.") The label may be a moiety such as a radioisotope, electrochemical tag, fluorescent tags, energy transfer (ET) labels, mass spectrometry tags, Raman tags, hapten, chemilluminescent group, enzyme, chromophore, and two or more labels. The label may also be charged, e.g. Cy5.5, bis-sulfonated carboxyfluorescein, or a dye attached to a charged moiety, e.g., carboxyfluorescein attached to cysteic acid or similar charged species. Methods for making these and other compounds are disclosed in U.S. Provisional Application No. 60/098,469 filed on August 31, 1998, and U.S. Application No. 90/018,695 filed on February 4, 1998, and PCT/GB98/00978 filed on April 2, 1998 and published on October 8, 1998, the disclosures of each application incorporated in their entirety by reference herein.

The terminator molecule is substituted with a moiety which imparts a net negative charge or a net positive charge to structure (I) at physiological or nucleic acid sequencing conditions. The moiety may be any charged species which alters the electrophoretic mobility of structure and degradation products, e.g., α-sulfo-β-alanine, cysteic acid, sulfonic acids, carboxylates, phosphates, phosphodiesters, phosphonates, amines, quaternised amines, and phosphonium moieties. As shown in Figure 2, the moiety (referred to as a "mobility modifier") may be attached to the terminator molecule between the linker and label (Figure 2a), between the base and linker (Figure 2b), and may be attached only to the sugar (Figure

2e) or only to the linker (Figure 2 c). The terminator molecule may also contain multiple linkers and moieties which are alternatively spaced together (Figure 2d). Although the moiety may be attached only to the base, it is believed that the presence of a charged moiety at this position may affect adversely affect the reactivity of the terminator molecule. The moiety may also be made of a number of charged units covalently linked together.
The charge-modified nucleic acid terminators, which along with the corresponding nucleic acid terminator decomposition products, migrate on separation media at different rates than the sequencing reaction products and result in improved sequence data (i.e., no blobs which obscure true data) and permit direct loading of nucleic acid sequencing reactions onto separating media.

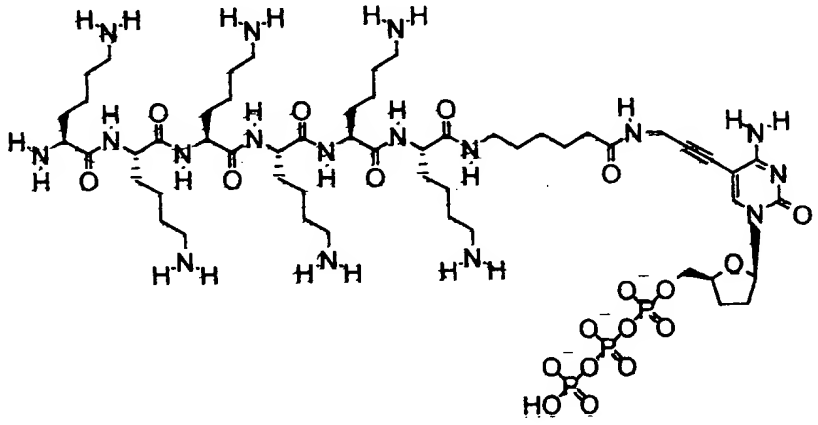
In particular, it has been found that the charge-modified nucleic acid terminators work especially well in sequencing reactions which also contain polymerases such as ThermoSequenase, which is Taq Δ271/ F272M/F667Y. The full length enzyme was truncated to eliminate 5' to 3' exonuclease activity, and to provide a polypeptide more stable to proteolysis and heat treatment. Therefore position 1 (amino acid Met) in ThermoSequenase corresponds to position 272 in full length Taq polymerase. Preferred polymerases include ThermoSequenase in which an amino acid substitution has been introduced at E410 (the numbering is for ThermoSequenase, not for Taq polymerase). An especially preferred polymerase is ThermoSequenase containing an E410R, E410W, or E410M substitutions. Such polymerases are described in PCT/US00/22150, the entire disclosure of which is incorporated herein by reference.

Additional preferred polymerases include a full length version of Taq polymerase with the following substitutions : D18A/E681R/F667Y. In this enzyme, the D18A substitution removes the 5' to 3' exonuclease activity, rather than the deletion of amino acids as in the ThermoSequenase polypeptide. The E681R substitution is the position equivalent to E410R in ThermoSequenase, and F667Y is the equivalent position to F396Y in ThermoSequenase. This enzyme also has properties desirable for sequencing with dye terminators. The amino acid sequence of Taq D18A/E681R/F667Y DNA polymerase. Such polymerases are also described in PCT/US00/22150, the entire disclosure of which is incorporated herein by reference.

The charge-modified nucleic acid terminators may have other important applications. They may also be useful in the therapeutic field as antiviral agents (anti-HIV and anti-HBV

etc) (WO 98/49177) and anticancer agents. Many nucleoside and nucleotide analogues have been developed as antiviral agents. They often act by inhibition of DNA polymerase and/or reverse transcriptase activity by a number of means. A number of nucleoside analogues, such as AZT, ddC, ddI, D4T, and 3TC are being used alone or in combination of other nucleoside or non-nucleoside analogues as anti-HIV agents. The charge-modified nucleic acid terminators of the present invention may also have antiviral activities alone or in combination with other compounds. Since combination drug therapy is being used more frequently to treat viral infections, having an increased number of compounds available by including compounds of the present invention could enhance the possibility of successful treatments.

The instantly charge-modified terminators could be transported into a cell in either the positively charged state, or if negatively charged, in a dephosphorylated state (which would then convert to a phosphorylated state in the cell) or the phosphate groups could be masked to facilitate entry into a cell and the masking groups later removed. One embodiment of a charge-modified terminator according to the instant disclosure which may be used in antiviral applications is



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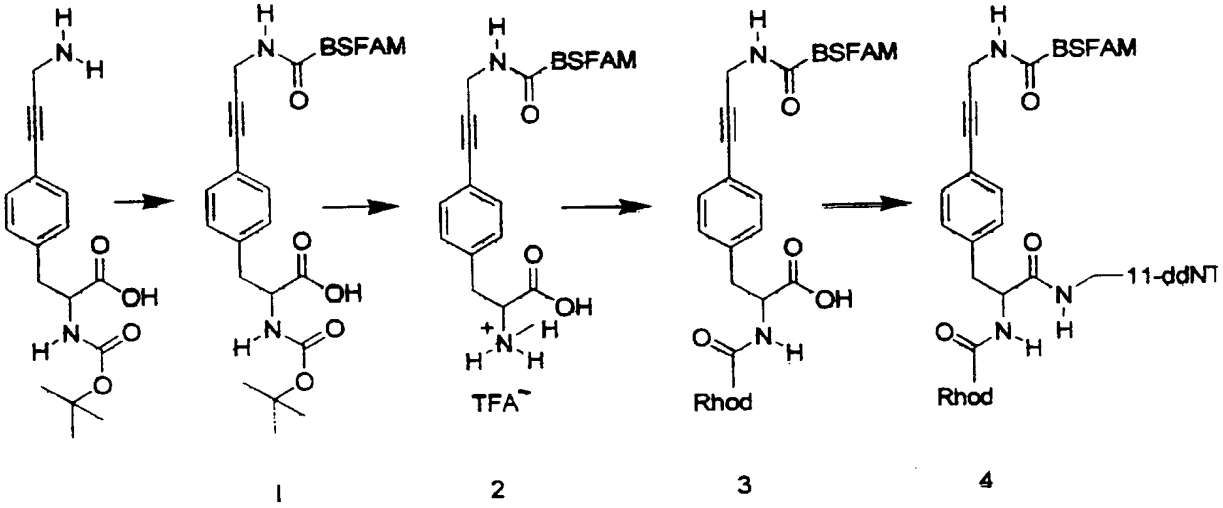
The following examples are for illustration purposes only and should not be used in any way to limit the appended claims.

EXAMPLES

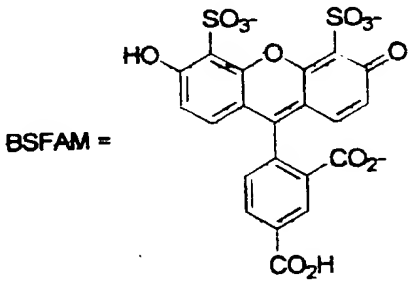
1. An example of charge modified reporters

1.1 Chemistry

The following scheme was used to synthesize labeled ddNTPs with a charged reporter moiety. The linker was synthesized according to methods disclosed in U.S. Provisional Application No. 60/098,469 filed on August 31, 1998, the entire disclosure of which is hereby incorporated by reference herein.



1 2 3 4



Rhod = 5-R110, 5-ROX, 5-TAMRA, 5-REG

1.2 Discussion

4',5' Bis-sulfono-5-carboxyfluorescein was attached to 4-propargylamino-N-α-*t*-butoxycarbonylphenylalanine by initial formation of the corresponding N-hydroxysuccinimide active ester using TSTU in DMF/N,N-diisopropylethylamine. Activation times were typically 15 minutes as observed by tlc before addition of the amino component. The product 1 was isolated by C18 RP-HPLC then treated with neat trifluoroacetic acid to remove the carbamate moiety, with the product 2 isolated by Et_2O precipitation. Attachment of the rhodamine dye was carried out using 5-rhodamine hydroxysuccinimde active esters in DMSO/N,N-diisopropylethylamine. All the double dye cassettes were purified by reverse phase HPLC prior to conjugation to alkylamino ddNTPs using published methods (and as disclosed in methods disclosed in U.S. Provisional Application No. 60/098,469 filed on August 31, 1998, the entire disclosure of which is hereby incorporated by reference herein.). The labeled ddNTPs were purified by silica gel chromatography followed by ion exchange chromatography then reverse phase HPLC.

1.3 Experimental

All chemicals were purchased from Sigma, Aldrich, Fluka or Fisher Scientific unless stated. UV/visible spectra were recorded on a Perkin Elmer Lambda 20 UV/visible spectrophotometer in conjunction with Winlab™ software. Prep HPLC was carried out on a Waters LC 2000 or LC 4000 system on a C18 Deltapak 15μm C18 100A 50x300mm column. Ion exchange chromatography was carried out on a Waters LC 600 system.

4-(propargylamido-4',5'-bissulfofluorescein)-N-α-*t*-butoxycarbonylphenylalanine (1)

4'-5'-bissulfo-5-carboxyfluorescein (100mg, 0.18mmol) was dissolved in DMF (4ml) then N,N-diisopropylethylamine (0.48ml, 15 eq.) and TSTU (65mg, 1.2eq.) added. The reaction mixture was stirred at room temperature for 1h. then 4-propargylamino-N-α-*t*-butoxycarbonylphenylalanine (69mg, 1.0eq) added. Stirring was continued for 3h. then the reaction mixture evaporated to dryness *in vacuo*. The product was isolated by reverse phase HPLC (C18, DeltaPak 15μ, 100A, 50x300μm) eluting with 0-100% eluant B over 60 min (A

= 0.1M TEAB, B = 50% MeCN/0.1MTEAB v/v, 100ml/min.). The product (retention time 37 min.) was evaporated to dryness *in vacuo* then coevaporated with MeOH (3x10ml) before lyophilization (100mg, 65%). UV/vis (1M triethylammonium bicarbonate pH 8.8) 495nm (24670), 465nm (shoulder, 9634), 312nm (6708).

4-(propargylamido-4',5'-bissulfofluorescein)-phenylalanine-α-ammonium trifluoroacetate (2)

4-(propargylamido-4',5'-bissulfofluorescein)-N-α-*t*-butoxycarbonylphenylalanine (100mg, 0.12mmol) was treated with trifluoroacetic acid (10ml) for 15min. then evaporated to dryness *in vacuo*. The residue was coevaporated with toluene (3x10ml) then the product precipitated by the addition of Et_2O (50ml). The solid formed was collected by filtration, washed with cold Et_2O (3x50ml) then dried under high vacuum (100mg, 99%). Rf (tlc, iPrOH:NH_4OH:H_2O (6:3:1)=0.

General methodology for the attachment of rhodamine dyes to 2 (3)

4-(propargylamido-4',5'-bissulfofluorescein)-phenylalanine-α-ammonium trifluoroacetate 2 (0.1mmol) was dissolved in DMSO (1ml) then N,N-diisopropylethylamine (0.26ml, 15 eq.) and rhodamine-NHS active ester (1.5 eq.) added. The reaction mixture was stirred at room temperature for 16h, then evaporated to dryness *in vacuo*. The R110, analog was treated with triethylammonium bicarbonate solution (0.1M, 50ml) for 16h to remove the trifluoroacetimido protecting groups. The products were purified by RP-HPLC using identical conditions to 1 unless stated. Retention times (BSFAM/R110 = 31min, BSFAM/R110 = 55min 0-100% B over 90 min, 100 ml/min., BSFAM/REG 54min 0-100%B over 90 min., 100ml/min, BSFAM/TAMRA = 52min 0-100% B over 90 min). All absorption spectra show the presence of both dyes.

General Methodology for Attachment of 3 to alkynlamino-2',3'-dideoxynucleotide triphosphates (4).

The double dye cassette (10.0 μmol) was dissolved in DMF (1ml) then disuccinimdyl carbonate (8 eq.) added as a solid at room temperature. The reaction was cooled to –60°C then DMAP (4 eq) in DMF (0.5ml) added. The reaction mixture was warmed to –30°C then a solution of aminoalkynl-ddNTP (0.67eq., Na_2CO_3/$NaHCO_3$ pH 8.5) added. The reaction was stirred at room temperature for 1h. then applied directly to a SiO_2 gel column. The product was eluted with iPrOH:NH_4OH:H_2O (4:5:1 v:v:v) then evaporated to near dryness *in vacuo* before subsequent purification by ion exchange chromatography then C18 reverse phase HPLC as for 1. Absorption spectra of each compound showed the presence of both dyes.

1.4 Comparative Electropherograms

Figure 3 provides an example of the increase in migration rate relative to sequence products of unincorporated bis-sulfofluorescein energy transfer terminators (and thermal breakdown products thereof) compared to the migration rate of the regular ET terminators.

2. An example of a negatively charged linker arm

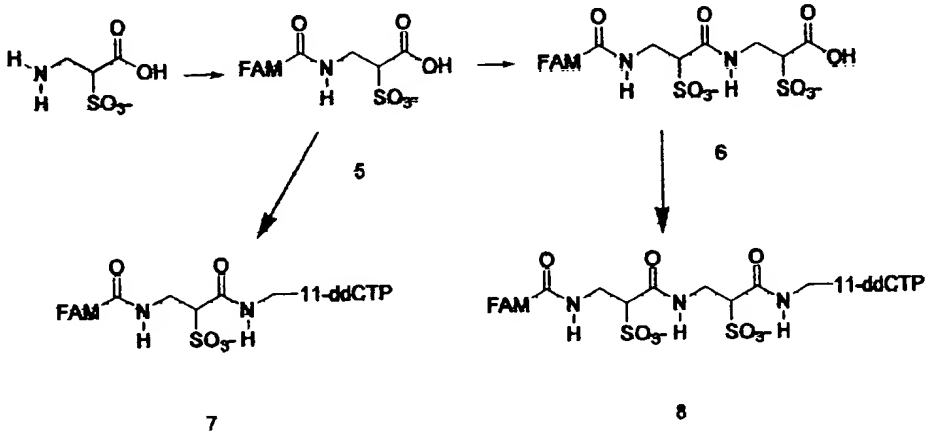
2.1 Background

By incorporation of a number of charged amino acids into the linker arm it is possible to synthesize a labeled ddNTP containing extra negative charge that alters the mobility of the degradative by-products observed in a sequencing reaction.

2.2 Chemistry

In order to determine the amount of negative charge required to remove colored by-products from the sequence ladder, fluorescein was attached to α-sulfo-β-alanine to form 5. Compound 5 was attached to 11-ddCTP (11=number of atoms in linker arm between nucleotide and dye) to form 7. A portion of 5 was attached to a second α-sulfo-β-alanine moiety to form 6 which was subsequently attached to 11-ddCTP to form 8. A control ddNTP

containing regular FAM attached to 11-ddCTP was also synthesized. The structures were run in a single color sequencing reaction to determine the effect of the charge on mobility.



As fluorescein carries a net 1- charge, compound 7 is considered as overall 2- linker arm, compound 8 has an overall 3- linker arm charge.

2.3 Experimental

N-5-carboxamidofluorescein-α-sulfo-β-alanine (5)

α-sulfo-β-alanine (59mg, 0.35mmol) was dissolved in DMF (2ml) then N,N-diisopropylethylamine (0.9mol, 15eq) added followed by 5-FAM-NHS active ester (200mg, 1.2eq.) The reaction mixture was stirred at room temperature for 3h. then evaporated to dryness *in vacuo*. The residue was coevaporated with MeOH (10ml) then the product isolated by C18 RP HPLC (A=0.1MTEAB, B=0.1MTEAB, 50%MeCN v/v) eluting with 0-100%B over 90 min at 100ml/min. ^{1}H nmr (300MHz, CD_3OD); 1.27(t, 24H, J=8.4Hz, NCH_2CH_3), 3.05(q, 16H, J=8.4Hz, NCH_2CH_3), 3.95-4.05(m, 3H, CH_2+$CHSO_3$), 6.58(m, 3H, Ar-H), 6.85(d, 2H, J=11.0Hz, Ar-H), 7.30(d, 2H, J=11.0Hz, Ar-H), 8.02(d, 1H, J=7.6Hz, ArH), 8.45(s,1H,Ar-H).

N-(N-5-carboxamidofluorescein-α-sulfo-β-alanine)amido-α-sulfo-β-alanine (6)

N-5-carboxamidofluorescein-α-sulfo-β-alanine (5, 50mg, 0.095mmol) was dissolved in DMF (3ml) then N,N-diisopropylethylamine (0.25ml, 15eq.) and TSTU (42mg, 1.5eq.) added. The reaction mixture was stirred at room temperature for 1h. then α-sulfo-β-alanine (24mg, 1.5eq.) added. Stirring was continued for 3h. then the reaction evaporated to dryness *in vacuo*. The product was isolated by ion exchange chromatography (mono-Q column, A=0.1M TEAB, 40%MeCN v/v, B=1.0M TEAB, 40%MeCN v/v, 0-50%B over 22min., 50-75%B from 22-50min. 75-100%B from 50-70 min., 4ml/min., retention time = 75-80min.) then C18 RP HPLC (A=0.1M TEABB=0.1M TEAB/MeCN 50% v/v, 0-100%B over 90 min., 100ml/min, retention time = 33min.). Rf (iPrOH6:ammonia3:water1v/v/v) 0.34.

General Methodology for Attachment of modified dyes to alkynlamino-2',3'-dideoxynucleoside triphosphates (7,8).

The modified dye (1mmol) was dissolved in DMF (5ml) then disuccinimdyl carbonate (4eq.) and DMAP (4eq.) were added at –60°C. The reaction mixture was stirred at –30°C for 15 min. then a solution of aminoalkyl-ddNTP (0.67eq., Na_2CO_3/$NaHCO_3$ pH 8.5) added. The reaction was stirred at room temperature for 1h. then applied directly to a SiO_2 gel column. The product was eluted with iPrOH:NH_4OH:H_2O (4:5:1 v:v:v) then evaporated to dryness *in vacuo* before subsequent purification by ion exchange chromatography then C18 reverse phase HPLC as for 1.

2.4 Results

Each labeled ddNTP was used in a single color sequencing reaction using standard sequencing protocols to generate a sequence ladder. Interpretation of the electropherograms shown in Figure 4 provided the conclusion that an overall 3- charge i.e compound 8 removed the colored by-products from the electropherogram. Figure 4 illustrates how the net negative charge of the dye labeled dideoxynucleotides effects their (and thermal breakdown products thereof) migration rate. As the net negative charge of the terminator increases, the migration rates of the various peaks seen (each of the peaks seen are either dye labeled dideoxynucleotides or thermal breakdown products thereof) increases (figure 4). At an

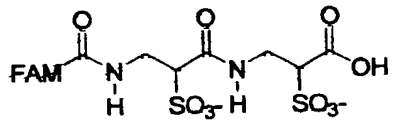
overall 3- charge (2- from linker, 1-from fluorescein) peaks are absent from the region of the electropherogram where sequence data would normally be obtained.

3. Negatively charged extended linker arms

3.1 Background

In order to improve the efficiency of incorporation of the modified terminator, a labeled terminator with a 3- charge on the linker arm was synthesized, this time containing an extended linker arm of 18 and 25 atoms.

3.2 Chemistry



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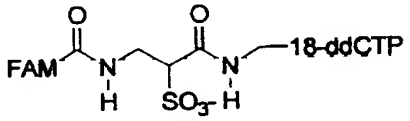

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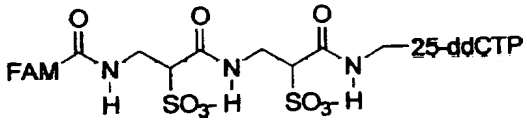

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3.3 Experimental

Compound 6, was attached to 18-ddCTP and 25-ddCTP using the standard protocol for attachment of labels to ddNTPs outlined in section 2.3. The method of purification was the same for 9 and 10.

Retention time of 9: Mono-Q ion exchange (47min)

Retention time of 10: Mono-Q ion exchange (42min)
C18 RP-HPLC (15min)

3.4 Sequencing Results

From the sequencing experiments it was clear that increasing the linker arm length improved incorporation of the terminator. This information, coupled to the presence of the 3- charge in the dye-linker structure led us to investigate rhodamine dyes with a 3- charged linker. This would permit four color sequencing. As shown in Figure 5, it is possible to directly load a sequencing reaction with no clean-up procedure. No peaks resulting from unincorporated dye-labeled terminator are observed in the sequence, demonstrating the utility of negatively charged with respect to direct load sequencing.

3.5 Rhodamine Labeled Terminators Containing a 3- Linker Arm

The following chemistry was attempted to synthesize a four colored set of terminators

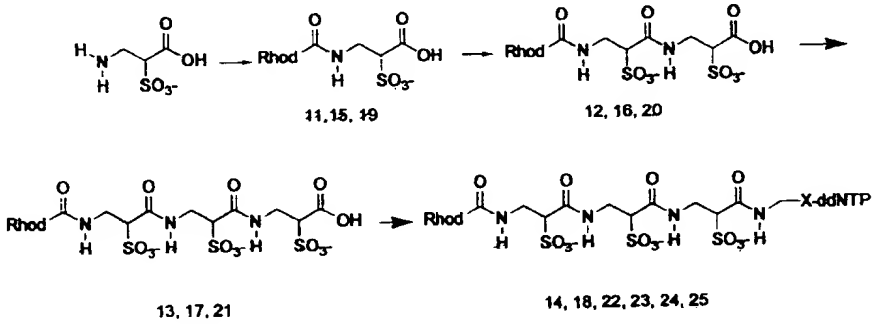


TABLE I

Compound Nos.	Rhod	X	N
11-14	REG	25	U
15-18	TAMRA	25	A
19-22	ROX	25	G
23	TAMRA	11	A

24	ROX	11	G
25	ROX	18	G

Rhod = rhodamine label, X = length of linker arm, N=base

3.6 Experimental

Compounds 11, 15, 19 were synthesized according to the method outlined for 5.
Compounds 12, 13,16,17,17,21 according to the method outlined for 6.
Compounds 14, 18,22-25 according to the general methodology for attachment of modified dyes to alkylamino-2',3'-dideoxynucleotide triphosphates (7,8).

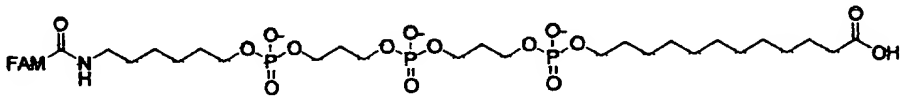
3.7 Results and Discussion

The labeled triphosphates 14, 18, 22 were used in direct load sequencing experiment. Compound 14 in a direct load experiment showed no breakdown products and with TSII and TaqERDAFY. Compounds 18 and 22 gave very dark sequencing bands and were observed to be forming an unexpected aggregate (as observed in the emission spectrum). The compounds also produced large colored blobs on a sequencing gel which interfered with interpretation of the sequence.

In order to overcome the aggregation effect, structures 23-25 were synthesized to investigate the effect of a shorter linker arm. Compound 23 has been shown to yield a clean sequence.

4 Other examples of negatively charged linker arms

Other negatively charged linker arms have been synthesized and studied for example the phosphodiester structure shown below. The product was synthesized using phosphoramidite chemistry however it could also be synthesized via H-phosphonates, phosphoroimidazolides, or phosphotriester chemistry.

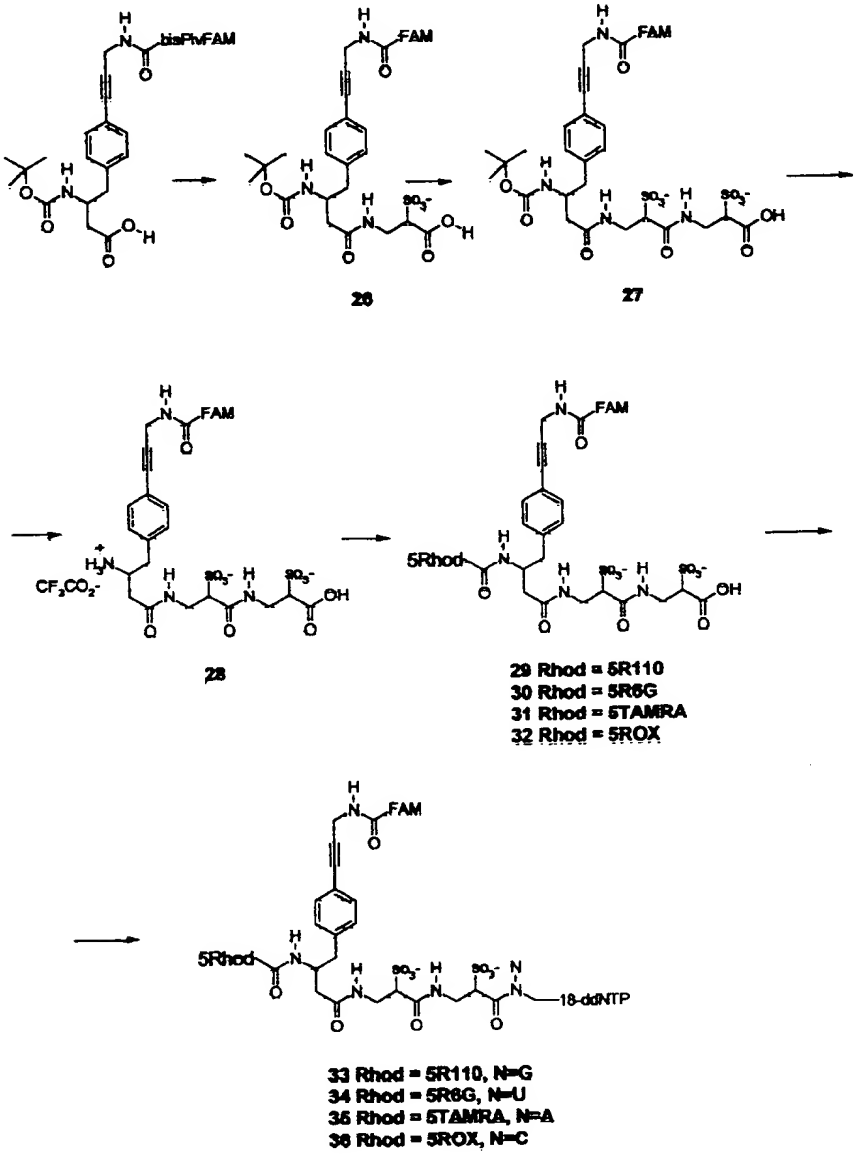


5. Examples of energy transfer labeled negatively charged linker arms

5.1 Background

In order to increase signal intensity a range of negatively charged, energy transfer labeled nucleotides were synthesized.

5.2 Chemistry



The starting material was synthesized according to the ET terminator patent and both α and β phenylalanine can be used in this chemistry. The single dye amino acid was reacted with α-sulfo-β-alanine in DMF/DMSO to yield a compound of formal two minus charge. The yield of compound **26** was improved by adding α-sulfo-β-alanine as a solution in DMSO. Compound **26** was isolated by ion exchange chromatography and the previous reaction repeated to yield compound **27**. The product was separated from starting material by ion exchange chromatography then the boc group removed by treatment with neat trifluoroacetic acid to yield compound **28**. The rhodamine dye was introduced by reaction of the amine in

aqueous buffer with a DMF solution of the rhodamine active ester. The products were isolated by ion exchange chromatography with any unreacted amine **28** recycled from the ion exchange column. The charged energy transfer molecules were conjugated to aminoalkynylnucleoside triphosphates using the DSC/DMAP method developed for energy transfer terminators and the products purified by silica gel chromatography, ion exchange separation then finally C18 RP HPLC.

5.3 Experimental

Boc-β-phenylalanine-4-(propargylamido-5-fluorescein)-α-sulfo-β-alanine (26)

Boc-β-phenylalanine-4-(propargylamido(bis-pivaloyl)-5-fluorescein) (400mg, 0.47mmol) was dissolved in DMF (4ml) then DIPEA (0.25ml, 3eq.) added followed by O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate (155mg, 1.1eq.) in DMF (1ml). The reaction was stirred at room temperature for 5min then a solution of α-sulfo-β-alanine (95mg, 1.2eq.) in DMSO (3ml, dissolved by gentle heating) added at room temperature. The reaction was stirred at room temperature for 16h then evaporated to near dryness *in vacuo*. The residue was treated with NH_4OH for 2h., the volume of the solution reduced *in vacuo*, water (50ml) added and the product purified by ion exchange chromatography (Q sepharose) eluting with the gradient shown below. A = 0.1M TEAB / 40% MeCN (v/v), B = 1.0M TEAB / 40%MeCN (v/v) flow = 6ml/min. detect at 500nm,. (2.0 AUFS). Product eluted in 50% buffer B.

TABLE II

Time/min	%B
0	0
20	10
40	10
45	20
60	20
65	30

85	30
90	40
110	40

TLC $_{SiO2}$ Rf (iPrOH:NH_4OH:H_2O 6:3:1 v:v:v) = 0.45. ^{1}H nmr (300 MHz, CD_3OD); 1.39 (s, 9H, $C(CH_3)_3$) 2.27 (m, 2H, $PhCH_2$), 2.80-2.85 (m, 3H, $CHCH_2$), 3.45 (m, 1H, $CHCH_2$), 3.75-3.85 (m, 3H, $NHCH_2CHSO_3$), 4.05 (m, 1H, $CHCH_2$), 4.43 (s, 2H, propargyl CH_2), 6.58 (s, 4H, FAM H1', H1'', H2', H2''), 7.00 (d, 2H, J=11.0Hz, Ar-H), 7.20 (d, 2H, J=11.0Hz, phenylalanine Ar-H), 7.38 (d, 3H, J=11.0Hz, 1 x FAM Ar-H,+ , 2 x phenylalanine Ar-H), 8.06 (d, 1H, J=7.6Hz, FAM H6), 8.45(s, 1H, FAM-H4).

N-(Boc-β-phenylalanine-4-(propargylamido-5-fluorescein)-α-sulfo-β-alanine)amido-α-sulfo-β-alanine (27)

Boc-β-phenylalanine-4-(propargylamido-5-fluorescein)-α-sulfo-β-alanine (100mg, 0.12 mmol) was dissolved in DMF (3ml) then DIPEA (0.1ml, 3eq.) added followed by O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate (50mg, 1.1eq.) in DMF (1ml). The reaction was stirred at room temperature for 5min then a solution of α-sulfo-β-alanine (25mg, 1.2eq.) in DMSO (1ml, dissolved by gentle heating) added at room temperature. The reaction was stirred at room temperature for 16h then evaporated to near dryness *in vacuo*. The residue was dissolved in water (50ml) and the product purified by ion exchange chromatography (Q sepharose) eluting with the gradient shown below. A = 0.1M TEAB / 40% MeCN (v/v), B = 1.0M TEAB / 40%MeCN (v/v) flow = 6ml/min. detected at 500nm,. (2.0 AUFS). Product eluted in 100% buffer B.

TABLE III

Time/min	%B
0	0
20	0
25	10
45	10

50	20
70	20
75	30
95	30
100	40
120	40
125	50

TLC $_{SiO2}$ Rf (iPrOH:NH_4OH:H_2O 6:3:1 v:v:v) = 0.25. ^{1}H nmr (300 MHz, CD_3OD); 1.39 (s, 9H, $C(CH_3)_3$) 2.27 (m, 2H, $PhCH_2$), 2.80-2.85 (m, 3H, $CHCH_2$), 3.45 (m, 1H, $CHCH_2$), 3.75-4.20 (m, 6H, 2x$NHCH_2CHSO_3$), 4.05 (m, 1H, $CHCH_2$), 4.43 (s, 2H, propargyl CH_2), 6.58 (s, 4H, FAM H1', H1'', H2', H2''), 7.00 (d, 2H, J=11.0Hz, Ar-H), 7.22 (d, 2H, J=11.0Hz, phenylalanine Ar-H), 7.387 (d, 3H, J=11.0Hz, 1 x FAM Ar-H,+ , 2 x phenylalanine Ar-H), 8.04 (d, 1H, J=7.6Hz, FAM H6), 8.47(s, 1H, FAM-H4).

N-(β-phenylalanine-4-(propargylamido-5-fluorescein)-α-sulfo-β-alanine)amido-α-sulfo-β-alanine (28)

N-(Boc-β-phenylalanine-4-(propargylamido-5-fluorescein)-α-sulfo-β-alanine)amido-α-sulfo-β-alanine (100mg, 0.10mmol) was treated with trifluoroacetic acid (10ml) for 1h. then the reaction evaporated to dryness *in vacuo*. The residue was triturated with Et_2O (30ml), the mother liquor was decanted from the yellow solid which was then dried under high vacuum.

General methodology for the attachment of rhodamine dyes to **28 (29, 30, 31, 32)**

N-(β-phenylalanine-4-(propargylamido-5-fluorescein)-α-sulfo-β-alanine)amido-α-sulfo-β-alanine (20.0μmol) was dissolved in $NaHCO_3$/Na_2CO_3 buffer (0.1M, pH 8.5, 3ml) then the desired rhodamine-NHS active ester (1.5 eq.) in DMF (3ml) added. The reaction mixture was stirred at room temperature for 16h, then evaporated to dryness *in vacuo*. The R110, analog was treated with triethylammonium bicarbonate solution (0.1M, 50ml) for 16h to remove the trifluoroacetimido protecting groups then the product purified by ion exchange chromatography. The other reaction mixtures were applied directly to a Q-sepharose ion

exchange column and eluted with the following gradient. A = 0.1M TEAB / 40% MeCN (v/v), B = 1.0M TEAB / 40%MeCN (v/v) flow = 6ml/min. detected at 500nm, (2.0 AUFS). Products eluted in 90% buffer B. Unreacted compound 28 eluted from the column in 100% buffer B which could be recycled in later reactions.

TABLE IV

Time/min	%B
0	0
10	10
35	10
60	90
90	100

Visible Absorption Spectra data for charged ET cassettes

5R110–βF-5FAM-(αsβala)$_2$-OH 445nm (1.02A), 510nm(0.72A) (TFA/H_2O solvent)

5R6G–βF-5FAM-(αsβala)$_2$-OH 498nm (1.14A), 525nm (0.85A)

5TAMRA–βF-5FAM-(αsβala)$_2$-OH 499nm (0.79A), 555nm (0.62A)

5ROX–βF-5FAM-(αsβala)$_2$-OH 498nm(0.83A), 595(0.60A)

General Methodology for Attachment of Modified Dyes to Alkynlamino-2',3'-dideoxynucleoside Triphosphates (33, 34, 35, 36).

The charged ET cassette (16.0 μmol) was dissolved in DMF (1ml) then disuccinimdyl carbonate (8 eq.) added as a solid at room temperature. The reaction was cooled to –60°C then DMAP (4 eq) in DMF (0.5ml) added. The reaction mixture was warmed to –30°C then a solution of aminoalkynl-ddNTP (0.67eq., Na_2CO_3/$NaHCO_3$ pH 8.5) added. The reaction was stirred at room temperature for 1h. then applied directly to a SiO_2 gel column. The product was eluted with iPrOH:NH_4OH:H_2O (4:5:1 v:v:v) then evaporated to dryness *in vacuo* before subsequent purification by ion exchange chromatography then C18 reverse phase HPLC as for compound 1.

5.4 Other dye labeled, negatively charged nucleoside triphosphates

Further negatively charged terminators have been synthesized using the chemistry outlined for single dye and energy transfer direct load terminators

5ROX–βF-5FAM-(αsβala)$_2$-18-ddGTP (37)

5FAM-(αsβala)$_2$-18-ddGTP (38)

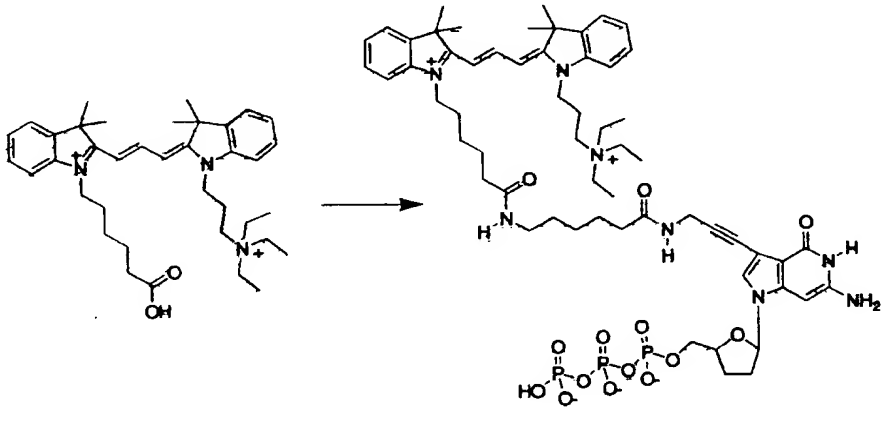
5.5 U.V. Visible Absorption Spectra of Compounds 33-36

All samples were analyzed in TE buffer, pH 8.5. The absorption spectra of compounds 33-36 are found at Figures 6-9.

6. Examples of Terminators with Formal Positively Charged Reporters

6.1 Background

In order to study positively charged structures, the following labeled terminator was synthesized.



39 40

6.2 Experimental

Compound 39 (10mg, 0.0134mmol) was dissolved in DMF (1ml) then N,N-diisopropylethylamine (23µl, 10eq.) added followed by PyBOP (14mg, 2.0eq.). The reaction mixture was stirred at room temperature for 15min. then a solution of 11-ddGTP (0.0083mmol, Na_2CO_3-$NaHCO_3$ pH 8.5) added in one portion. The reaction mixture was stirred at room temperature for 3h. then applied directly to a silica gel column. The product was eluted with iPrOH:NH_4OH:H_2O (6:3:1 v/v/v) then purified by ion exchange chromatography (as for 6) followed by C18 RP-HPLC (1.75µmol yield, 21%).

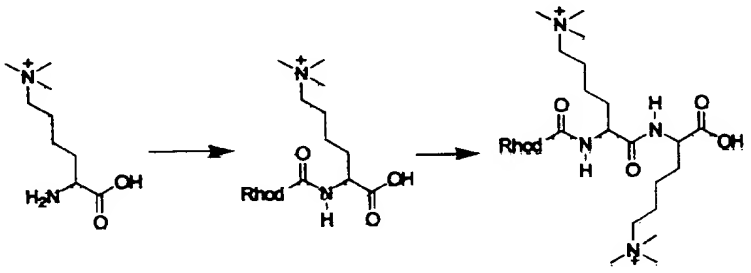
6.3 Sequencing Results

The electropherogram shown in Figure 10 was obtained when compound 40 was used in a sequencing reaction. The +2 charged terminator was used in a sequencing reaction and loaded directly on to a slab gel. The same experiment was repeated, however the reaction mixture was treated with phosphatase prior to loading on a gel to remove phosphates from the unincorporated dye-labeled dideoxynucleotides remaining in the reaction mixture. This would leave all terminator derived products with an overall positive charge causing them to migrate in the opposite direction to the sequence products during electrophoresis. It is clear from the electropherogram that the colored by-products are absent from the sequence when phosphatase is used to break down the terminator products.

7. Formal positive charged extended linker arms

7.1 Chemistry

Another example of dyes attached to a positively charged linker arm is shown below;



41 42

In this example, the rhodamine dye R6G is attached to ε-N,N,N-trimethyllysine which contains a formalized positive charge from the ε quaternary amine. The product (41) can be further modified to yield a +2 linker arm (42) by reaction with a further molecule of the charged amino acid. Further reaction(s) would generate the desired charged structure.

7.2 Experimental

α–N–(5–carboxamidorhodamine6G)-ε-N,N,N-trimethyllysine (41)

ε-N,N,N-trimethyllysine (68mg, 30.0mmol) was dissolved in DMF (6ml) then N,N-diisopropylethylamine (0.5ml, 10eq.) added followed by R6G-NHS active ester (200mg, 1.2eq.). The reaction mixture was stirred at room temperature for 16h. then evaporated to dryness *in vacuo*. The product was isolated by C18 RP HPLC (A=0.1M TEAB, B=0.1MTEAB/50%MeCN, 0-100%B over 50 min., 100ml/min). Retention time = 44min.

α–(α'–N–(5–carboxamidorhodamine6G)-ε'-N,N,N-trimethyllysine)-ε-N,N,N-trimethyllysine (42)

α–N–(5–carboxamidorhodamine6G)-ε-N,N,N-trimethyllysine 28 (100mg, 0.15mmol) was dissolved in DMF (5ml) then N,N-diisopropylethylamine (0.3ml, 15eq.) and TSTU (67mg, 1.5eq.) added. The reaction mixture was stirred at room temperature for 1h, then ε-N,N,N-trimethyllysine (50mg, 1.5eq.) added. The solution was stirred for a further 3h. then the reaction mixture was evaporated to dryness *in vacuo*. The product was isolated by C18 RP

HPLC (A=0.1M TEAB, B=0.1MTEAB/50%MeCN, 0-100%B over 90 min., 100ml/min). Retention time = 60min.

8 The Use of Amino Groups in the Linker Arm as Carriers of Positive Charge

8.1 Background

Compounds containing formal positive charge have numerous challenges associated with their chemical synthesis and purification. By using compounds containing amino functions, it may be possible to utilize protonation of the amino moiety to impart the desired charge. The synthetic problems of formal positive charge may also be overcome by using a suitable protecting group to mask the amino function(s). The protecting group can be removed in a straightforward manner at the end of the chemical synthesis.

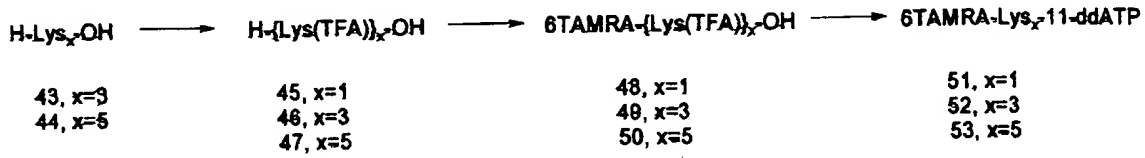
The initial molecule studied was lysine, which has a number of advantages.

2. The pKa of the ε-NH_2 function is approximately 10 hence the amino group will be charged at the pH of a typical DNA sequencing separation
3. There are a number of protecting groups available for lysine which can be removed without degrading other moieties in the desired target molecule
4. The number of lysines required can be readily modified using standard peptide synthesis methodologies (formal positively charged peptides containing ε'-N,N,N-trimethyllysine are synthetically extremely demanding)

A range of TAMRA labeled lysine oligomers were synthesized and conjugated to 11-ddATP to determine the following information

2. Whether the amino group protonates under the conditions used in the separation step of dideoxy DNA sequencing?
3. How many lysine residues are required to prevent dye-labeled breakdown products from interfering with sequence information ?

8.2 Chemistry



Commercially available oligolysines were selectively protected at the ε-amino group using the desired stoichiometric amount of ethyl trifluoroacetate at 4°C. The major product was isolated by C18 RP-HPLC and confirmed to be the desired product by electrospray mass spectrometry. The free amino group was labeled using 6-TAMRA-NHS active ester then the product attached to 11-ddATP using standard conjugation chemistry. Compounds 51, 52 and 53 were then used in a single color sequencing reaction and the products applied directly to a sequencing gel (ABD 377). As shown by the gel of Figure 11, increasing the number of lysine residues removes colored degradation byproducts from the sequence. Increasing the number of lysine residues is also believed to increase the reactivity of the substrate. Experiments in which the deoxynucleotide concentration was kept constant and the amount of dideoxynucleotide concentration decreased showed that lower amounts of ddNTP terminators continued to exhibit longer sequencing ladders indicating that lysine modified ddNTPs are better substrates for polymerase than conventional ddNTPs. Thus, it was concluded that the reactivity of the lysine ddNTPs increases in proportion the number of lysines present on the terminator molecule.

8.3 Experimental

General method for introduction of trifluoracetamido protecting group to e-NH_2 of oligo-lysine (46, 47)

Oligolysine (0.16mmol) was dissolved in MeOH (8ml) then triethylamine (133µl, 6eq.) added and the reaction cooled to 4°C. Ethyl trifluoroacetate (3eq for 46, 5eq for 47) was added and the reaction stirred at 4°C for 16h. The reaction was evaporated to dryness *in vacuo* and the product purified by C18 RPHPLC using the gradient shown below. The product containing fractions were evaporated to dryness *in vacuo* and the product precipitated

by the addition of Et_2O (50ml). The mother liquor was decanted and the solid dried under high vacuum.

Buffer A = Water/TFA (0.1% v/v), B = MeCN/TFA (0.1% v/v), 120 ml/min., 210nm detection.

TABLE V

Time/min	%B
0	0
20	0
80	100

General method for introduction of 6-TAMRA to protected lysine xmers (48,49,50)

Side chain protected lysine xmer (22.0μmol) was dissolved in DMF (2 ml) then N,N-diisopropylethylamine (35μl, 10eq.) added followed by 6-TAMRA-NHS (10mg, 1.3eq.) in DMF (1ml). The reaction was stirred at room temperature for 16h. then the product purified by C18 RPHPLC using the gradient shown below. The desired product had the longest retention time on C18 HPLC in all cases. The product containing fractions were evaporated to dryness *in vacuo* and the product precipitated by the addition of Et_2O (50ml). The mother liquor was decanted and the solid dried under high vacuum.

TABLE VI

Time/min	%B
0	0
20	0
80	100

Buffer A = Water/TFA (0.1% v/v), B = MeCN/TFA (0.1% v/v), 120 ml/min., 210nm detection.

General method for introduction of 6-TAMRA to protected lysine xmers (51,52,53)

TAMRA labeled protected lysines (48, 49, 50, 3.9 μmol) was dissolved in DMF (3ml) then N,N-diisopropylethylamine (0.3ml, 400eq.) added followed by O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate (10mg, 10eq.). The reaction was stirred at room temperature for 5 min. then cooled to 0°C before 11-ddATP (5mg) added in $NaHCO_3/Na_2CO_3$ (0.1M, pH 8.5, 2ml). The ice bath was removed immediately and the reaction stirred at room temperature for 30 min. The product was purified by silica gel chromatography, eluting with MeOH to remove starting materials then 6:3:1 (iPrOH: NH4OH:H2O v:v:v) to elute the desired TFA protected compound. The product containing fractions were combined then evaporated to near dryness *in vacuo* and the product purified by mono-Q ion exchange chromatography eluting with the following gradient. A = 0.1M TEAB / 40% MeCN (v/v), B = 1.0M TEAB / 40%MeCN (v/v) flow = 6ml/min. detected at 550nm, (2.0 AUFS).

TABLE VII

Time/min	%B
0	0
7	0
32	50
70	75
90	100

The product containing fractions were evaporated to dryness *in vacuo* then treated with NH_4OH (50ml) for 16h. The reaction mixture was evaporated to dryness then the product purified by C18 RPHPLC eluting with the following gradient. A = 0.1M TEAB B = 1MeCN flow = 25ml/min. detected at 550nm, (1.0 AUFS).

TABLE VIII

Time/min	%B
0	0
45	100

The product containing fractions were evaporated to dryness then the product dissolved in TE buffer pH 8.5.

The methodology was repeated to synthesize the remaining three labeled nucleotides shown below

5R110-Lys_5-11-ddGTP (54)
6R6G-Lys_5-11-ddUTP (55)
6ROX-Lys_5-11-ddCTP (56).

A number of other dye-(lysine pentamer) nucleotides were synthesized to determine the effect of linker arm length and are shown below

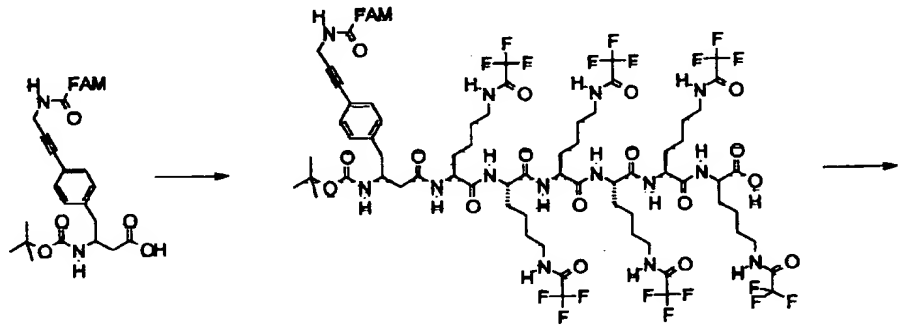
5R6G-Lys_5-18-ddUTP (57)
5FAM-Lys_6-11-ddGTP (58)

8.4 Four color sequencing using lysine labeled nucleotides

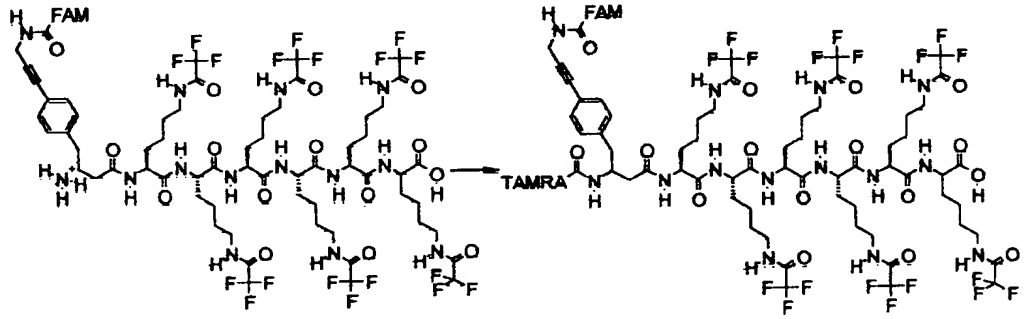
In Figure 12, the sequence in lane 2 was generated using compounds **53, 54, 55, 56.** As shown in Figure 12, unincorporated terminators comigrate with sequencing data (lane 1), positively charged rhodamine terminators according to the instant disclosure migrate backwards (lane 2), and negatively charged rhodamine terminators according to the instant disclosure migrate before sequencing data (lane 3).

8.5 Examples of energy transfer labeled polylysine nucleotides

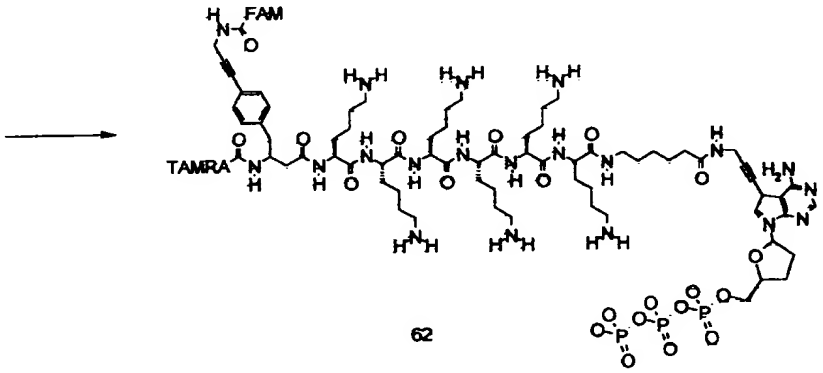
The synthesis of hexalysine labeled nucelotides with an energy transfer label is shown below



59



60 61



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8.6 Experimental

α-t-butoxycarbonyl-(4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine (59)

α-t-butoxycarbonyl-(4-progargylamido-5-fluorescein)-phenylalanine (50mg, 0.051mmol) was dissolved in DMF (2ml) then N,N-diisopropylethylamine (90µl, 10 eq.) added followed by O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate (23mg, 1.5 eq.) in DMF (1ml). The reaction was stirred at room temperature for 1h. then further O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate (8mg, 0.4eq.) added. The reaction was stirred at room temperature for a further 30min then the solution added to hexa-(ε-trifluoracetamido)lysine (77mg, 1.0eq.). The reaction was stirred at room temperature for 16h. then the product isolated by C18 HPLC eluting with the gradient as shown below. The product containing fractions were evaporated to near dryness *in vacuo*, then the suspension frozen and lyophilized (31mg, 30%).

Buffer A = water/TFA (0.1% v/v), B = MeCN/TFA (0.1% v/v), 120 ml/min., 210nm and 445nm detection. Product eluted at t=50 min. TOF MS ES+ found 2023.0 (M+Na+), theoretical $C_{86}H_{101}F_{18}N_{14}O_{22}$ 2023.7.

TABLE IX

Time/min	%B
0	0
7	0
32	50
70	75
90	100

(4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine (60)

α-t-butoxycarbonyl-(4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine (59, 30mg, 14.4µmol) was treated with trifluoroacetic acid (10ml) for 30 min. then evaporated to dryness *in vacuo*. The residue was treated with Et_2O (50ml) to afford a yellow solid. The supernatant liquor was decanted, the residue triturated with further portions of Et_2O (2 x 10ml) then dried under high vacuum. TOF MS ES+ found 1944.7 ($M+Na^+$), theoretical $C_{81}H_{91}F_{18}N_{14}O_{20}Na$ 1944.6.

α-N-5TAMRA-4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine (61)

(4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine (60, 100mg, 49.0μmol) was dissolved in DMF (1ml) and N,N-diisopropylethylamine (85μl, 10eq.) added. The solution was added to a solution of 5-TAMRA-NHS (50mg, 1.9eq.) and the reaction mixture stirred at room temperature for 16h. The product was purified by C18 RP HPLC, by elution with the following gradient;

Buffer A = water/TFA (0.1% v/v), B = MeCN/TFA (0.1% v/v), 120 ml/min., 550nm and 445nm detection. Product eluted at t=50 min.

TABLE X

Time/min	%B
0	0
30	0
40	40
100	80

The product eluted at 75 min and was observed to have absorptions at 445nm (lactone form of fluorescein) and 550nm(TAMRA absorption). The product containing fractions were combined and evaporated to dryness *in vacuo* and the residue triturated with Et_2O (100ml) before drying under high vacuum. TOF MS ES- found 2328.4 (M-H), theoretical $C_{106}H_{106}F_{18}N_{16}O_{24}$ 2328.7.

5-TAMRA-(4-progargylamido-5-fluorescein)-phenylalanine-hexalysine-11-ddATP conjugate (62)

α-N-5TAMRA-4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine (61, 10mg, 4.3μmol) was dissolved in DMF (1ml) then N,N-diisopropylethylamine (7μl, 10eq.) added followed by O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate (6mg, 5eq.). The reaction mixture was stirred at room temperature for 5

min. then cooled to 0° C before addition of 11-ddATP (0.3ml, 4mg, 1eq) in $NaHCO_3/Na_2CO_3$ (pH 8.5) buffer. The reaction was raised to room temperature and stirred for a further 1h. The product was purified by Q sepharose ion exchange chromatography using the gradient shown below;

Buffer A = 0.1M TEAB/MeCN (40% v/v), B = 1.0M TEAB/MeCN (40% v/v), 120 ml/min., 500nm and 550nm detection. Product eluted at t=38 min.

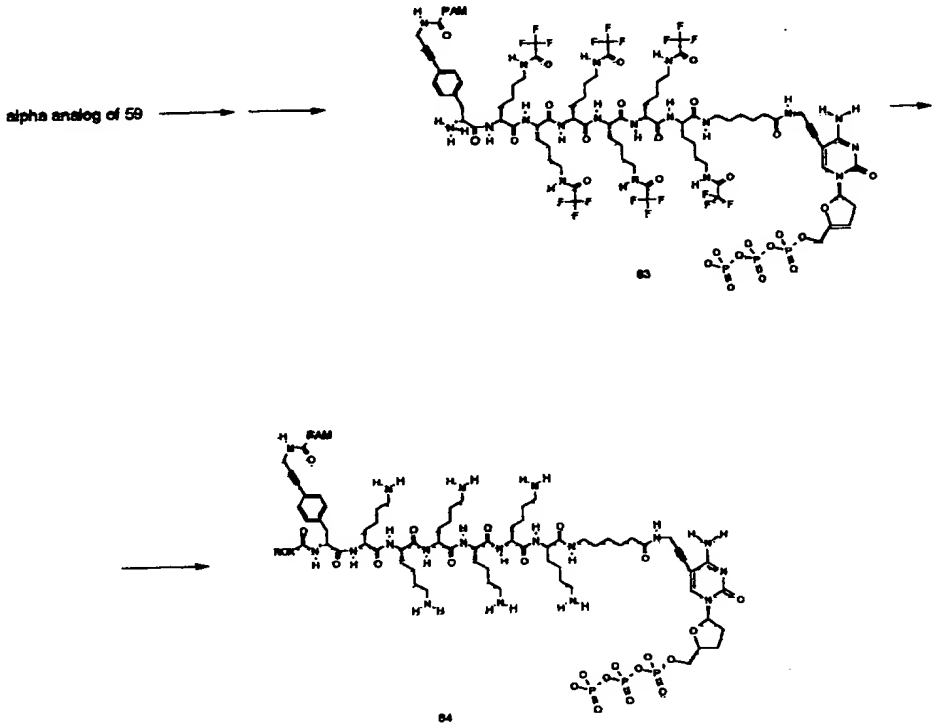
TABLE XI

Time/min	%B
0	0
7	0
60	100

The product containing fractions were evaporated to dryness *in vacuo* and the residure treated with NH_4OH (c. 10ml) for 16h. The reaction mixture was evaporated to near dryness *in vacuo* then the product repurified by Q sepharose ion exchange chromatography using the same gradient. The product containing fractions were evaporated to dryness *in vacuo* then dissolved in TE buffer prior to use in a sequencing reaction.

8.7 FAM ROX-Lys6-11-ddCTP synthesis

FAMROX-Lys_6-11-ddCTP was synthesized using the following pathway:



Compound 59 was attached to 11-ddCTP using O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate as the activating reagent. The product was deprotected as a crude mixture using neat trifluoroacetic acid for 20 min. then any unreacted 11-ddCTP removed from the reaction by C18 HPLC. The modified nucleotide was then dissolved in DMSO and reacted with a DMSO solution of 5-ROX-NHS active ester.

8.9 Experimental

(4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine-11-ddCTP conjugate (63)

α-t-butoxycarbonyl-(4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine (59, 30mg, 150μmol) was dissolved in DMF (1ml) then N,N-diisopropylethylamine (26μl, 10eq.) added followed by O-(N-succinimidyl)-N,N,N',N'-tetramethyleneuronium tetrafluoroborate (10mg, 2eq.) in DMF (0.5ml). The reaction was stirred at room temperature for 10 min., cooled to 0°C then 11-ddCTP (DMF solution, 5.8mM, 3ml, 1.2eq.) added. The solution was raised to room temperature and stirring

continued for 3h. The reaction was evaporated to dryness *in vacuo* and the residue triturated with Et_2O (50ml). The supernatant liquid was decanted and the solid dried under high vacuum. The residue was treated with TFA (15ml) for 20 min then evaporated to dryness *in vacuo*. The residue was dissolved in water (100ml) and remaining unreacted 11-dCTP removed by C18 HPLC. The product containing fractions were combined and evaporated to dryness *in vacuo*.

Buffer A = water/TFA (0.1% v/v), B = MeCN/TFA (0.1% v/v), 120 ml/min., 300nm and 445nm detection. Product eluted at t = 50 min.

TABLE XII

Time/min	%B
0	0
30	0
32	50

5-ROX-(4-progargylamido-5-fluorescein)-phenylalanine-hexalysine-11-ddCTP conjugate (64)

(4-progargylamido-5-fluorescein)-phenylalanine-hexa-(ε-trifluoramido)lysine-11-ddCTP conjugate (63, approx. 15μmol) was dissolved in DMSO (3ml) and N,N-diisopropylethylamine (52μl, 10eq.) added followed by 5-ROX-NHS (47mg, 75μmol) in DMSO (5ml). The reaction was stirred at room temperature for 16h. then the protected protected nucleotide purified by Q-sepharose ion exchange chromatography eluting with gradient A shown below followed by C18 RP-HPLC (gradient B). The desired fractions were evaporated to dryness *in vacuo* then the residue treated with NH_4OH (c., 200ml) for 16h. The reaction was concentrated to near dryness *in vacuo* then the product purified by Q sepharose ion exchange chromatography eluting with gradient A.

Gradient A, Buffer A = 0.1M TEAB/MeCN (40% v/v), B = 1.0M TEAB/MeCN (40% v/v), 120 ml/min., 500nm and 575nm detection. Product eluted at t=50 min.

TABLE XIII

Time/min	%B
0	0
7	0
60	100

Gradient B, Buffer A = 0.1M TEAB, B = MeCN, 120 ml/min., 500nm and 575nm detection. Product eluted at t = 38 min.

TABLE XIV

Time/min	%B
0	0
10	0
70	100

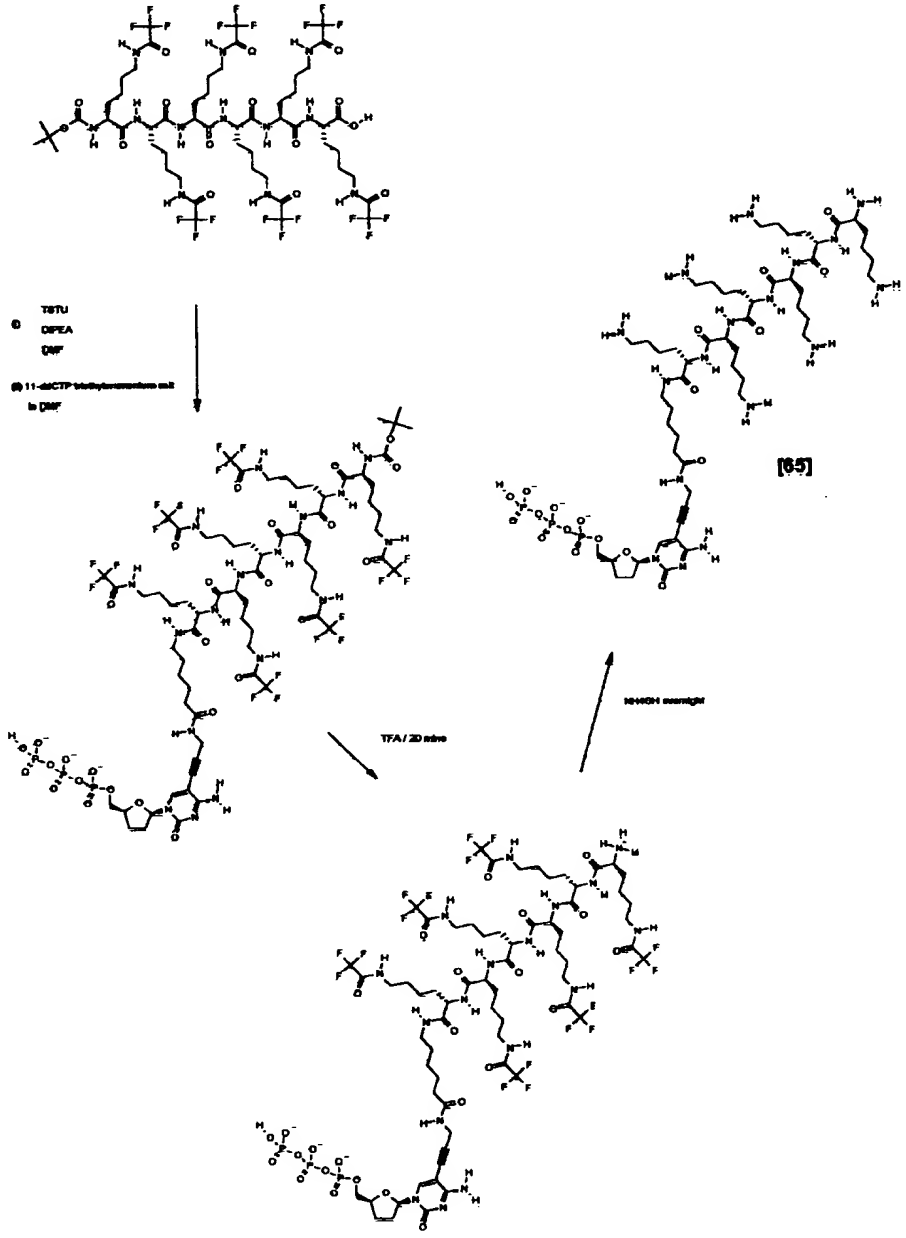
8.10 U.V. Visible Spectra of Compounds 62 and 64

Samples were analyzed in TE buffer, pH 8.5. UV/visible spectra for compounds 62 and 64 are shown in Figures 13 and 14.

9. Example of reporterless dideoxynucleotide diphosphate bearing positively charged groups

Synthesis of H-Lys_6-11-ddCTP [65]

H-Lys$_6$-11-ddCTP was synthesized as follows:



To a clean, dry vessel was added Boc-(Lys(TFA))$_6$-OH (33mg, 22.55μmoles) and this was dissolved in DMF (2ml). To this was added TSTU as a DMF solution (13.6mg, 45.05μmoles in 1ml) and DIPEA (0.226mmoles, 39μl). The vessel was swirled several times to ensure a colorless solution. After 1 hour, 11-ddCTP, as the triethylammonium salt, was added as a DMF solution (22.55μmoles, 3.9ml) and the solution swirled several more times.

3 hours after addition of ddNTP the reaction was reduced to a clear gum *in vacuo*. Addition of diethyl ether (20ml) and vigorous swirling caused the gum to form into flakes of white solid. The ether was then removed *via* pippette and the white flakes dried *in vacuo*. The flakes were then dissolved in neat trifluoroacetic acid (10ml), the solution swirled and left for 20 minutes. The acid was removed *in vacuo* to leave a clear oil, and more ether was added (20ml) to form white flakes of solid. This solid was dissolved in DMF (10ml) and loaded onto a preparatory C_{18} HPLC system with monitoring at 300nm and a flow rate of 100ml/min

TABLE XV

Time (mins)	%A (Water 0.1% v/v TFA)	%B (Acetonitrile 0.1% TFA)
0	100	0
30	100	0
90	0	100

Peak eluting between 47 and 50 minutes was collected and reduced to dryness *in vacuo*. Acetonitrile (50ml) was added and the mixture reduced to dryness again. This was repeated 2 more times until a white solid remained. Trituration was effected with ether and then the liquid removed *via* pippette. The solid was dried *in vacuo* before ammonium hydroxide was added (100ml). The solid did not immediately dissolve and so the mixture was stirred vigorously overnight. The next day, a clear solution remained which was then reduced to approximately one third volume *in vacuo*. Normal phase tlc of the remaining solution exhibited a 254nm spot, $R_f = 0$, with a mobile phase of 6:3:1 iPrOH : NH_4OH : H_2O.

Solution loaded onto APBiotech MonoS cation exchange column XK10 with deionised water. Monitoring at 300nm and flow of 5mlmin^{-1}.

Time (mins)	%A (0.1M TEAB 40% Acetonitrile pH 7-8)	%B (1M TEAB 40% v/v Acetonitrile pH 7-8)
0	100	0
5	100	0
65	0	100

Peak at 47-51 minutes collected and reduced to a clear gum *in vacuo*. Methanol (25ml) was added and the mixture reduced again. This was repeated twice further until a white solid remained. The white solid was dissoved in pH 8.5 Tris-EDTA buffer (2ml). Yield 1.86μmoles (8.2%).

Electrospray mass spectrometry, positive ion mode: $C_{54}H_{103}N_{17}O_{19}P_3$ m/z where z=2, 693.1, monosodium salt $C_{54}H_{103}N_{17}NaO_{19}P_3$ m/z where z=2, 704.6, trisodium salt $C_{54}H_{10}N_{17}Na_3O_{19}P_3$ m/z where z=2, 726.1.

Abbreviations

Abbreviation	Definition
ddNTP	2'-3'-dideoxynucleoside triphosphate
ET	Energy Transfer
TSTU	2-Succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate
PyBOP	Benzotrialzol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
DMF	N,N-dimethylformamide
RP HPLC	Reverse Phase High Performance Liquid Chromatography
Et_2O	Diethyl ether
DMSO	Dimethyl sulfoxide
TEAB	Triethylammonium bicarbonate
MeCN	Acetonitrile
iPrOH	Isopropanol
NH_4OH	Ammonium Hydroxide
BSFAM	4',5' Bis-sulfono-5-carboxyfluorescein
R110	Rhodamine 110
REG or R6G	Carboxyrhodamine6G
TAMRA	Tertamethylrhodamine
ROX	Carboxy-X-rhodamine
DMAP	4-dimethylaminopyridine
11-ddGTP	2',2'-dideoxyguanosine triphosphate with an 11 atom linker arm
NHS	N-hydroxysuccinimide

Although various embodiments of the instant invention are described in detail above, the instant invention is not limited to such specific examples. Various modifications will be readily apparent to one of ordinary skill in the art and fall within the spirit and scope of the following appended claims.

CLAIMS

What is claimed is:

1. A compound comprising structure (I)

Z—X—S—B—L (I)

wherein

Z is mono-, di or triphosphate or thiophosphate, or corresponding boranophosphate

X is O, CH_2, S, or NH;

S is a sugar or a sugar analogue;

B is a naturally occurring or a synthetic base;

L is alkyl, alkenyl, or alkynyl and is optionally substituted with a reporter moiety; and

L, B, S, X, or Z are substituted with a moiety which imparts a net negative charge or a net positive charge to structure (I) at physiological or nucleic acid sequencing conditions.

4. The compound according to Claim 1, wherein the moiety which imparts a net negative charge or a net positive charge to structure (I) is selected from the group consisting of α-sulfo-β-alanine, cysteic acid, phosphate, sulfate, sulfonate, carboxylate, phosphodiester, phosphonate, phosphonium, amine, and higher alkyl or aryl amines.

5. The compound according to Claim 3, wherein the moiety is a primary, secondary, tertiary, or quarternary amine.

6. The compound according to Claim 3, wherein the moiety is lysine.

7. The compound of Claim 1 wherein L is substituted with a reporter moiety.

8. The compound of Claim 1 wherein the reporter moiety is an energy transfer label.

9. The compound of Claim 1 wherein the linker contains up to about 100 atoms.

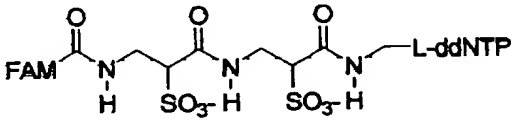
10. The compound of Claim 1 wherein the linker contains about 2 to about 50 atoms when structure (I) contains a net positive charge.

11. The compound of Claim 1 wherein the linker contains about 11 to about 25 atoms when structure (I) contains a net positive charge.

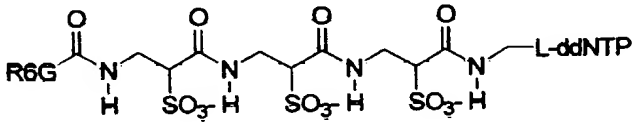
12. The compound of Claim 1 wherein the linker contains about 11 to about 25 atoms when structure (I) contains a net negative charge.

13. The compound of Claim 1 wherein the linker contains about 18 to about 25 atoms when structure (I) contains a net positive charge.

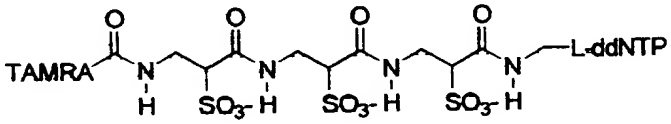
14. A compound selected from the group consisting of



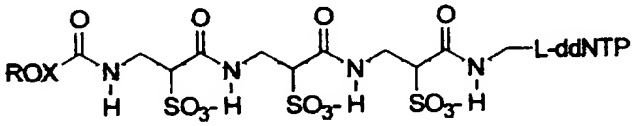
II,



III,



IV, and

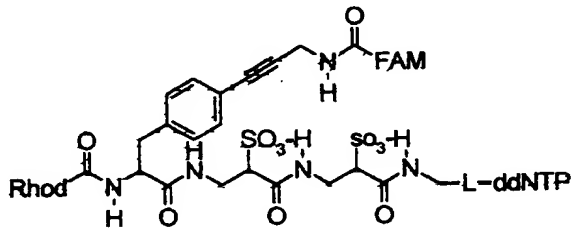


V.

wherein L is an alkyl, alkenyl, alkynyl containing more than ten atoms when N is a purine base or analog thereof and L is an alkyl, alkenyl, alkynyl containing more than twenty atoms when N is a pyrimidine base or analog thereof;

and isomers thereof.

15. A compound comprising structure (VI)



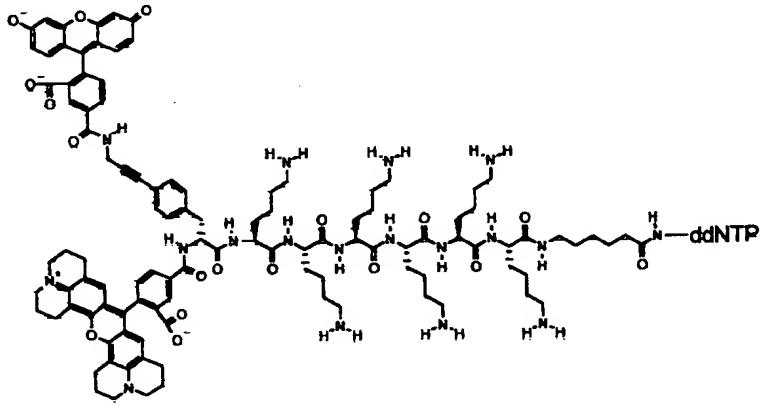
VI

wherein Rhod is selected from the group consisting of 5R110, 5R6G, 5TAMRA, 5ROX and isomers thereof;

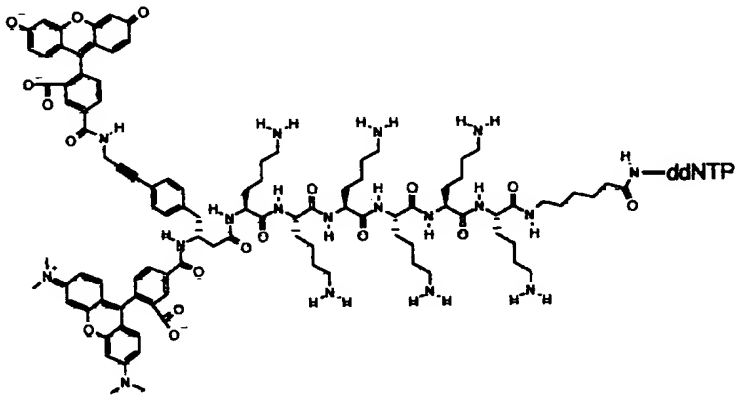
L is an alkyl, alkenyl, alkynyl containing more than four atoms,

and isomers thereof.

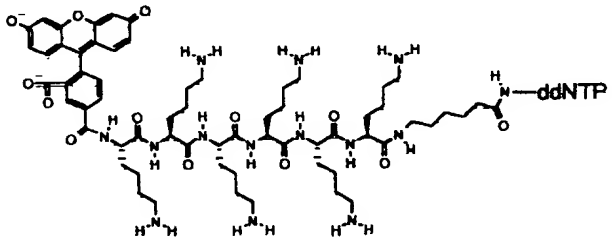
16. A compound selected from the group consisting of



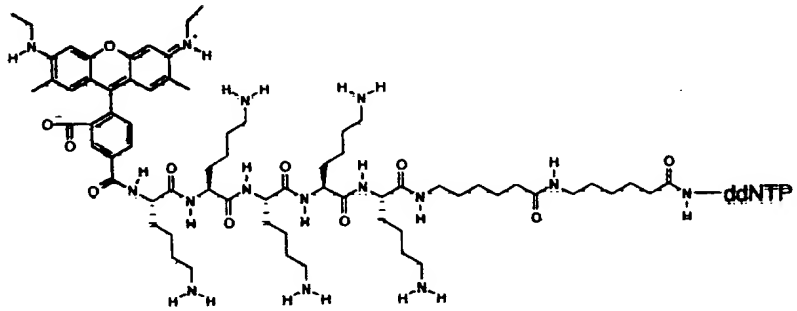
VII,



VIII,



IX, and



X

and isomers thereof.

17. A method for sequencing a nucleic acid that comprises:

A. reacting a nucleic acid template, nucleoside triphosphates or analogs thereof, and compound (I) according to Claim 1 with a polymerase to generate fragments,

B. electrophoretically separating said fragments, and

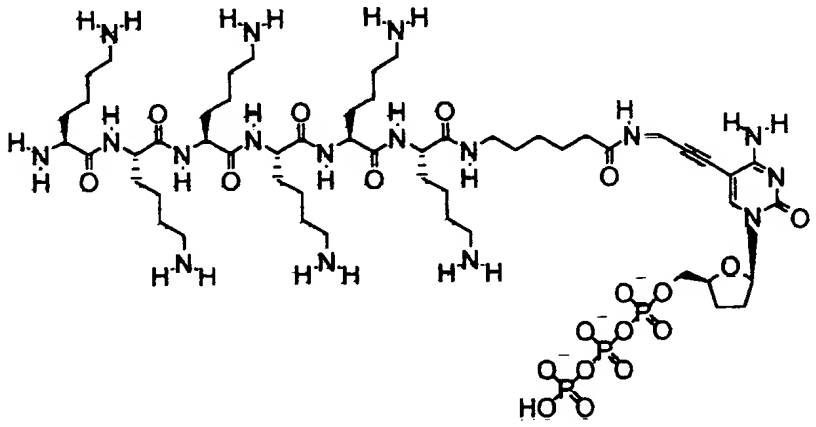
C. determining the sequence of the nucleic acid template.

18. A kit for sequencing nucleic acids comprising compound (I) of Claim 1.

19. A composition comprising compound (I) of Claim 1.

20. A method for inhibiting a virus that comprises contacting a cell infected with a virus with a virus-inhibiting effective amount of the compound of Claim 1.

21. A compound of formula XI



XI

and isomers thereof.

22. A method for inhibiting a virus that comprises contacting a cell infected with a virus with a virus-inhibiting effective amount of the compound of Claim 21.

23. The method according to Claim 20 wherein the compound of Claim 1 is administered orally, bucally, topically, intravenously, parentally, or rectally.

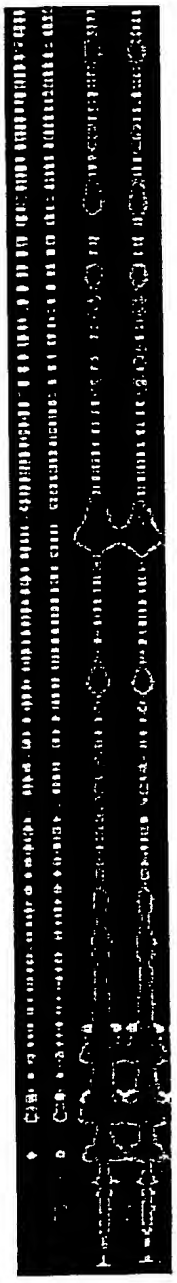


Fig. 1

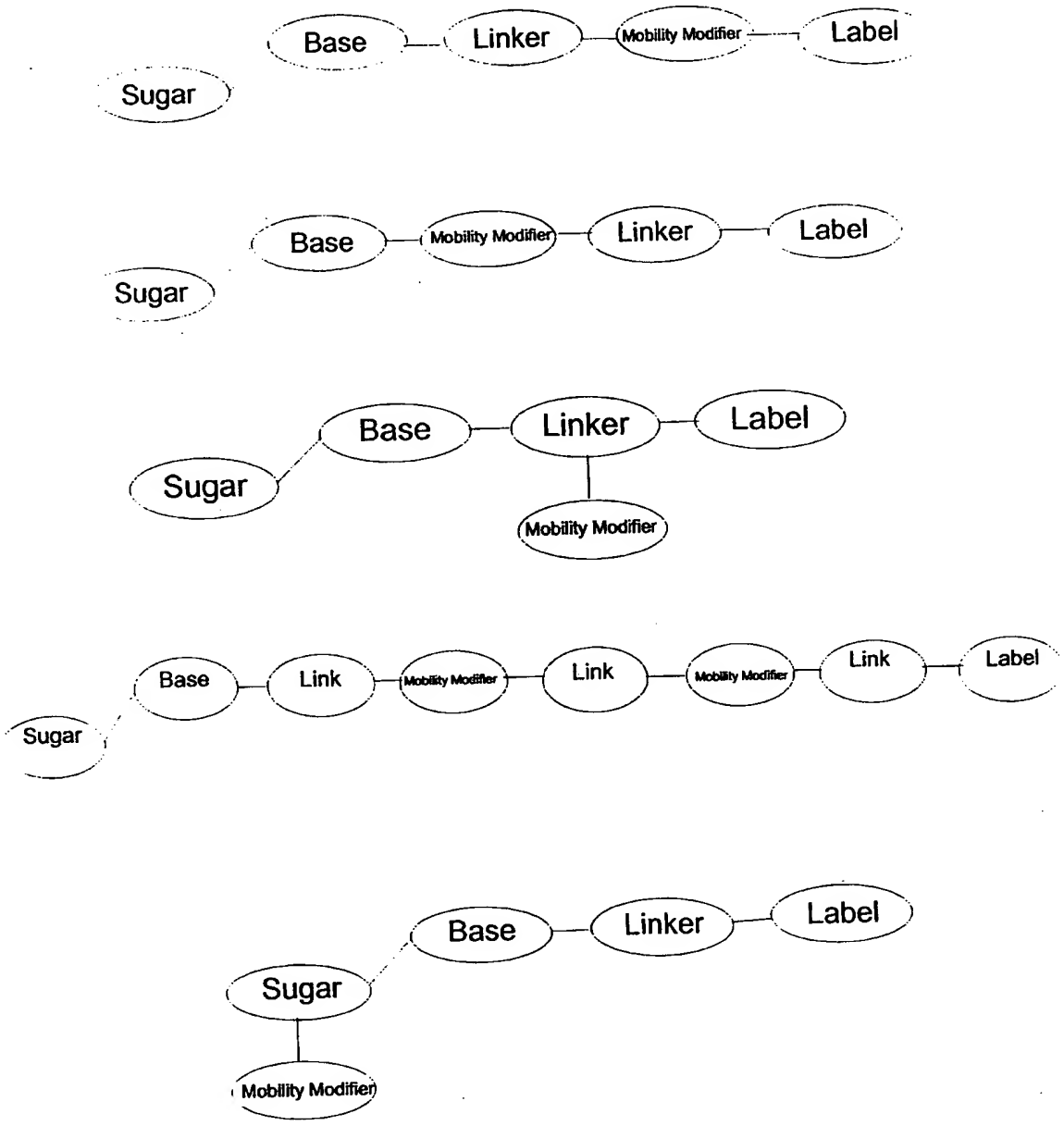

Base
Linker
Mobility Modifier
Label
Sugar
Base
Mobility Modifier
Linker
Label
Sugar
Base
Linker
Label
Sugar
Mobility Modifier
Base
Link
Mobility Modifier
Link
Mobility Modifier
Link
Label
Sugar
Base
Linker
Label
Sugar
Mobility Modifier

Fig. 2

normal energy transfer terminator

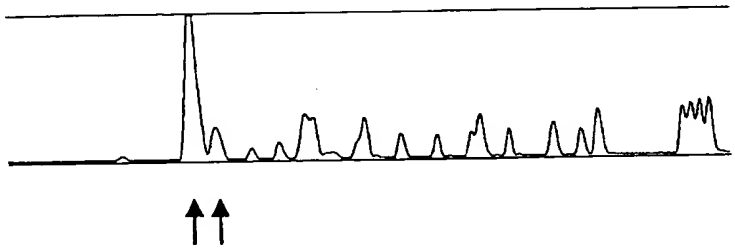


Fig. 3a

Bis sulfonated fluorescein energy transfer terminator

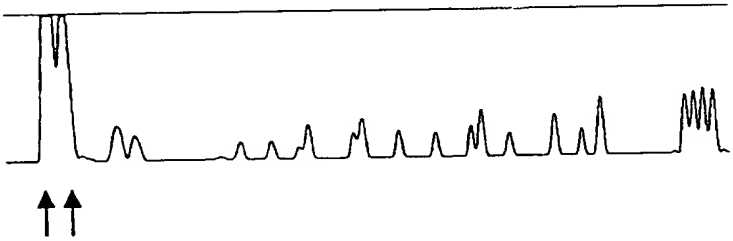
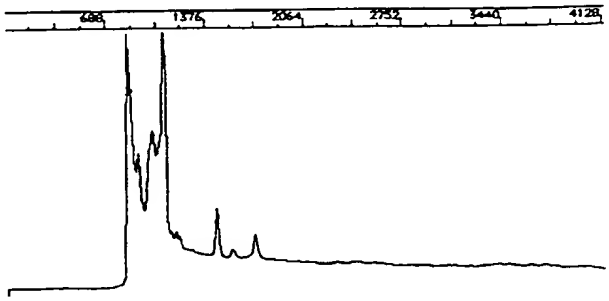


Fig. 3b

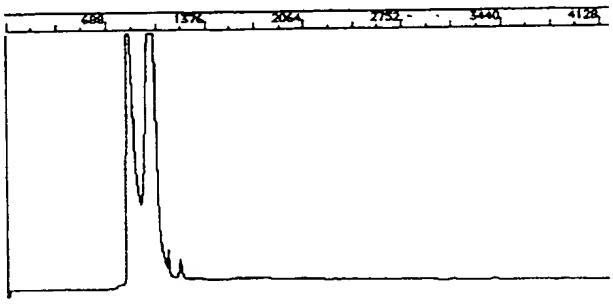
Faster Slower

Fig. 4a



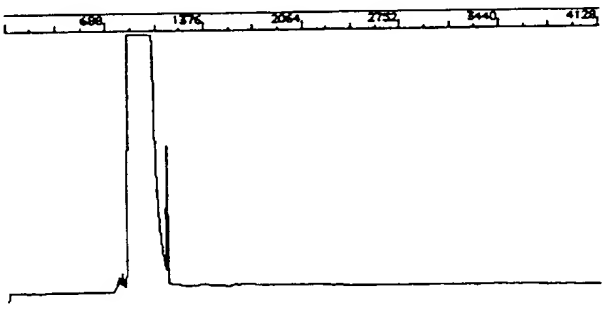
FAM-11-ddCTP

Fig. 4b



Compound 7

Fig. 4c



Compound 8

Faster Slower

Net -3 charge terminator (10) reaction, directly loaded

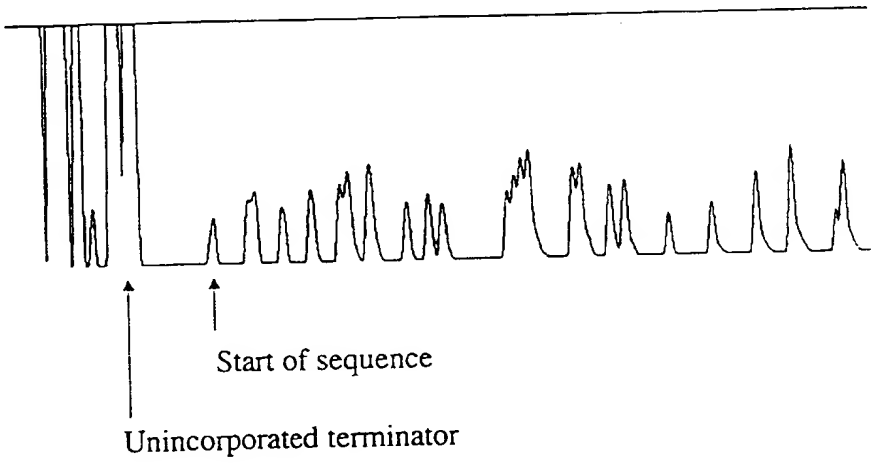
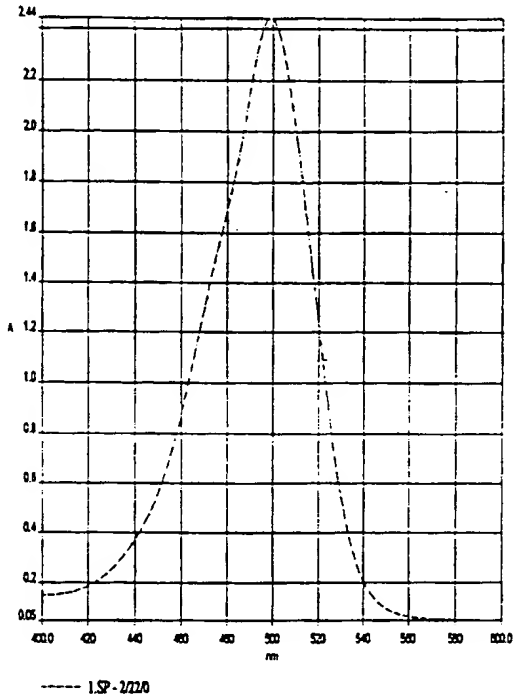
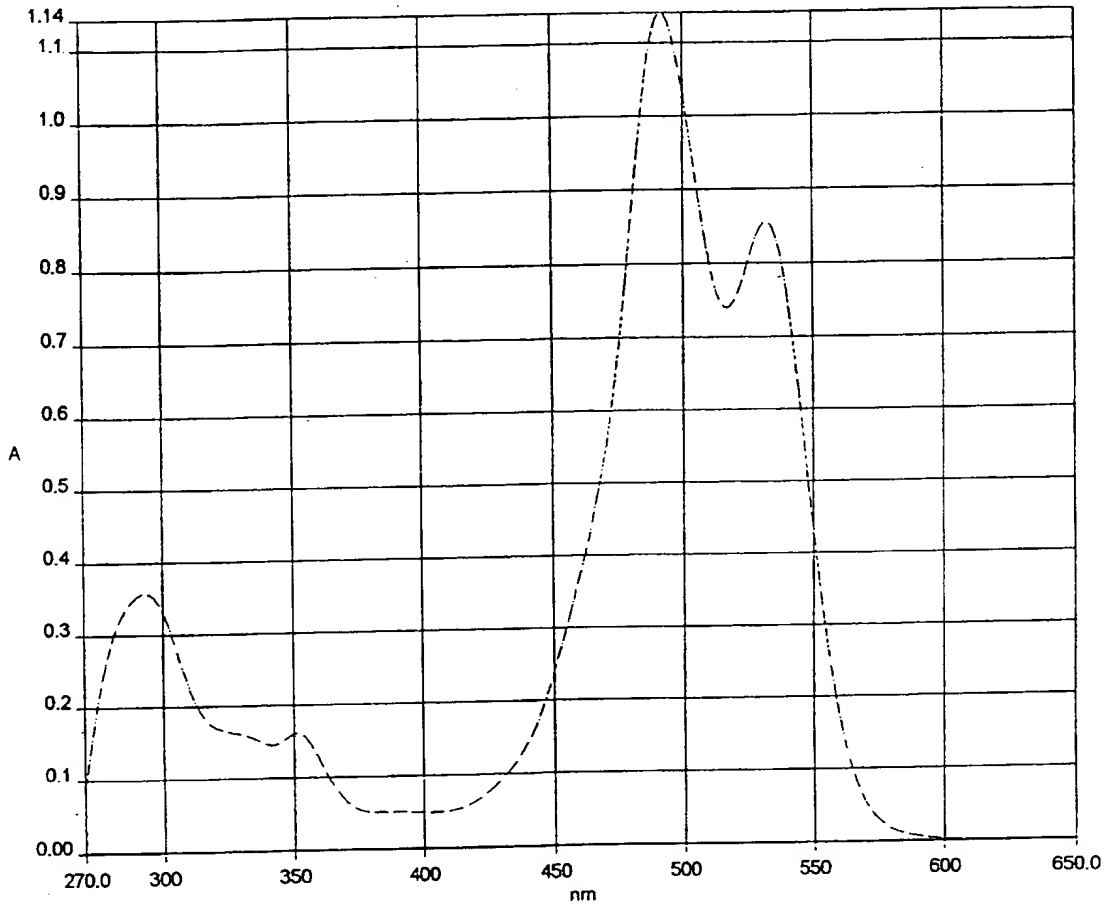


Fig. 5



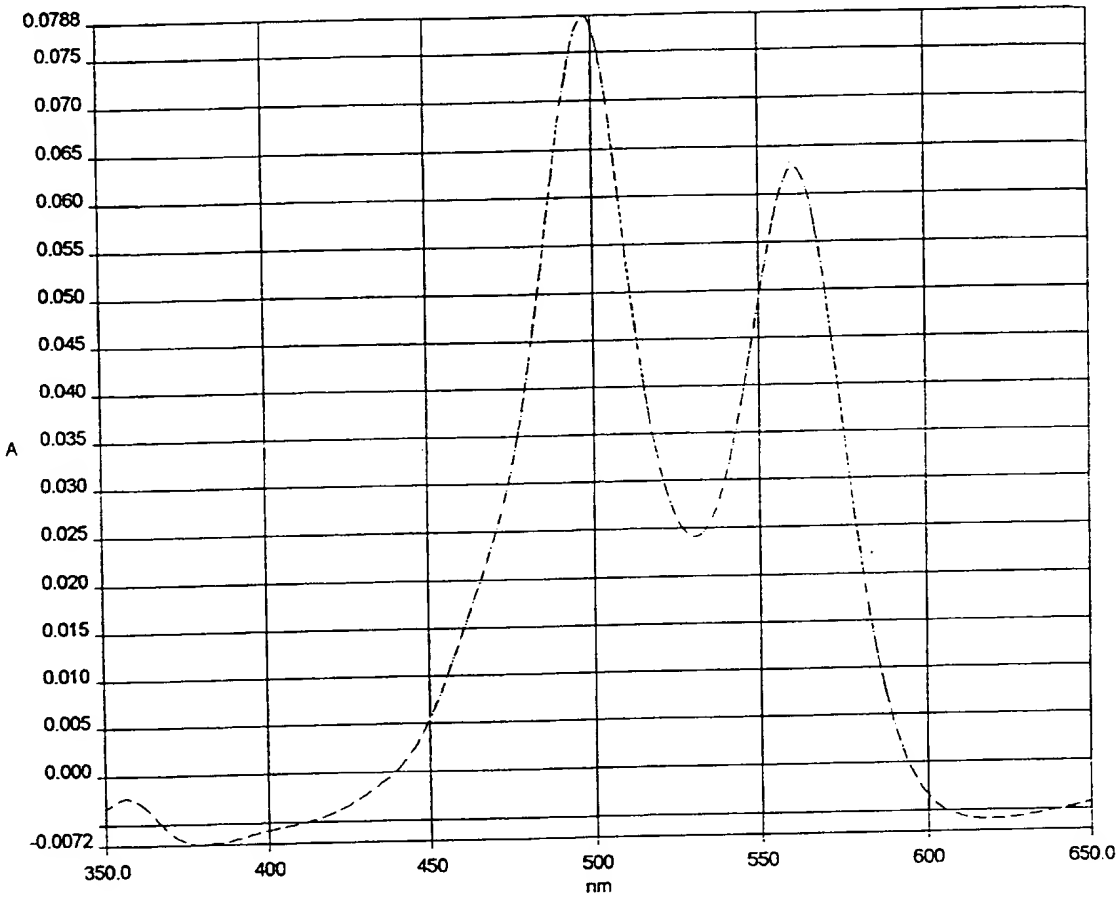
FAM-R110(3-)-18-ddGTP

Fig. 6



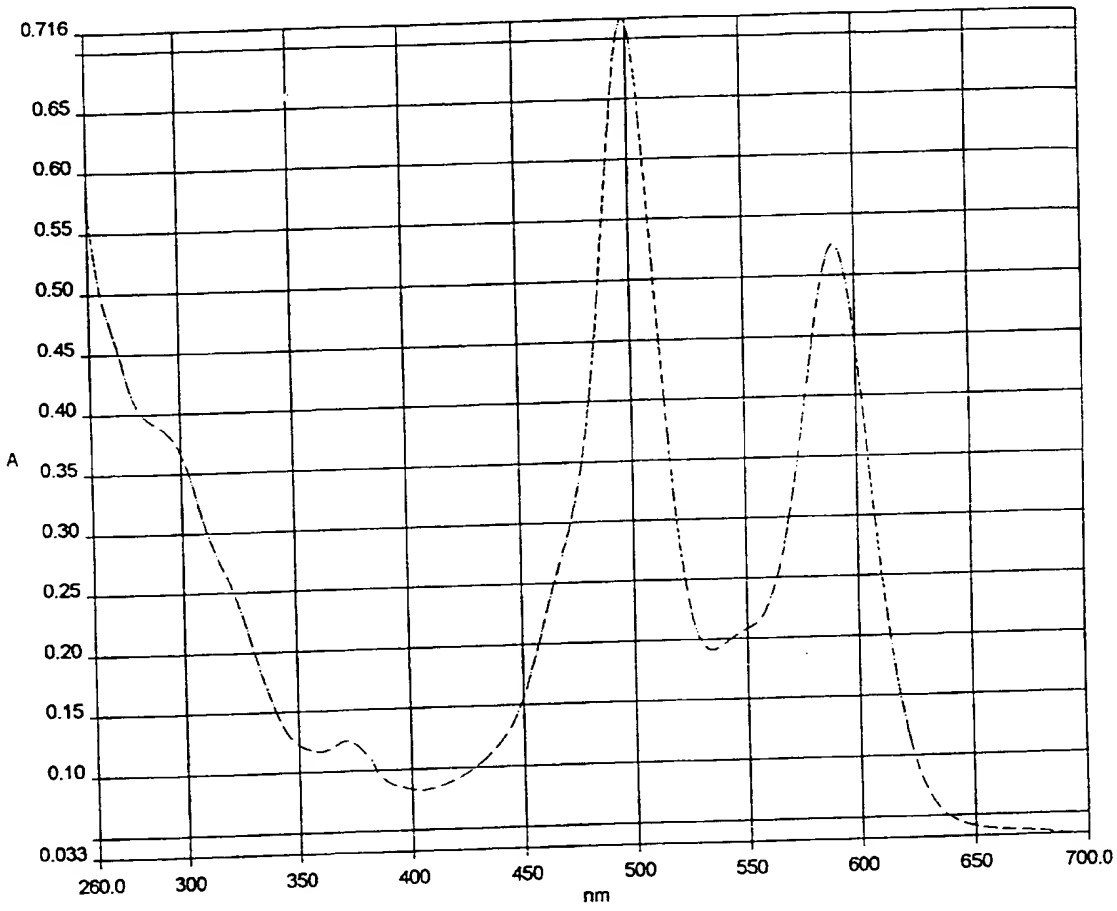
FAMR6G(3-)-18-ddUTP

Fig. 7



FAMTAMRA(3-)-18-ddATP

Fig. 8

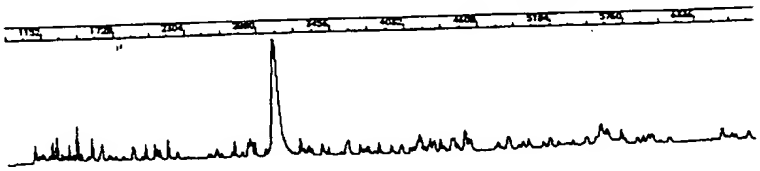


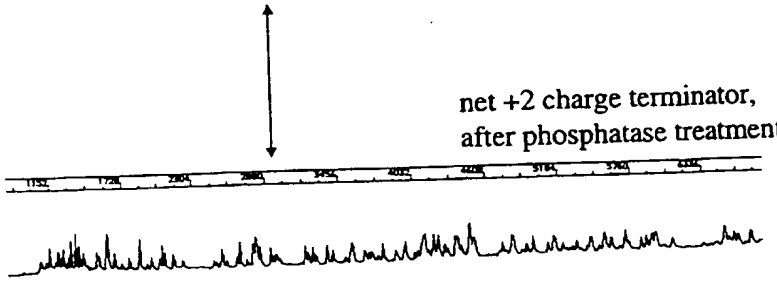
FAMROX(3-)-18-ddCTP

Fig. 9

net +2 charge terminator,
no phosphatase treatment

Fig. 10a





net +2 charge terminator,
after phosphatase treatment

Fig. 10b

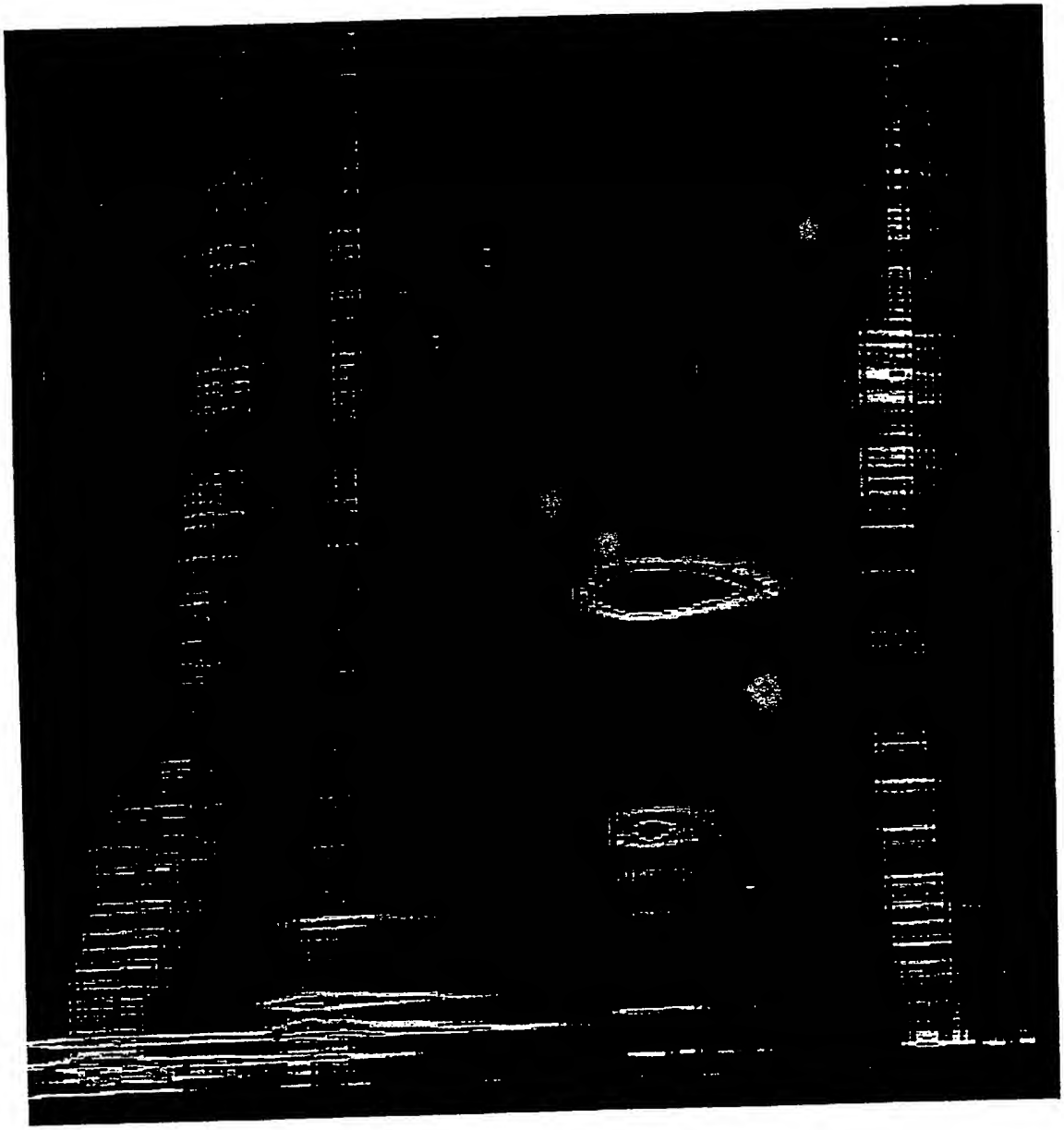


Fig. 11

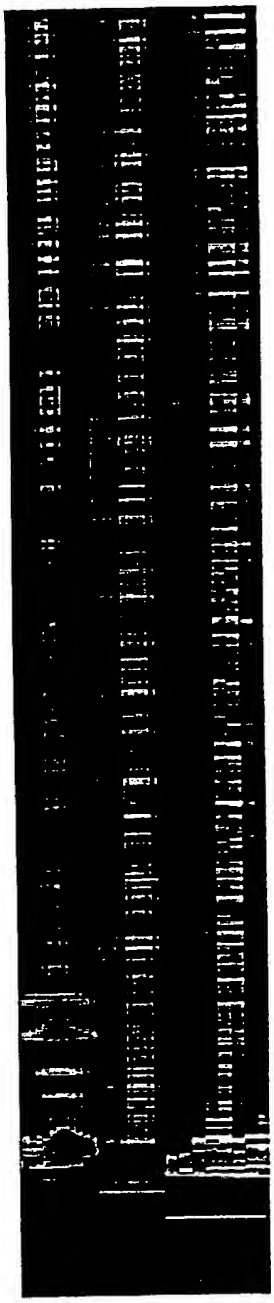
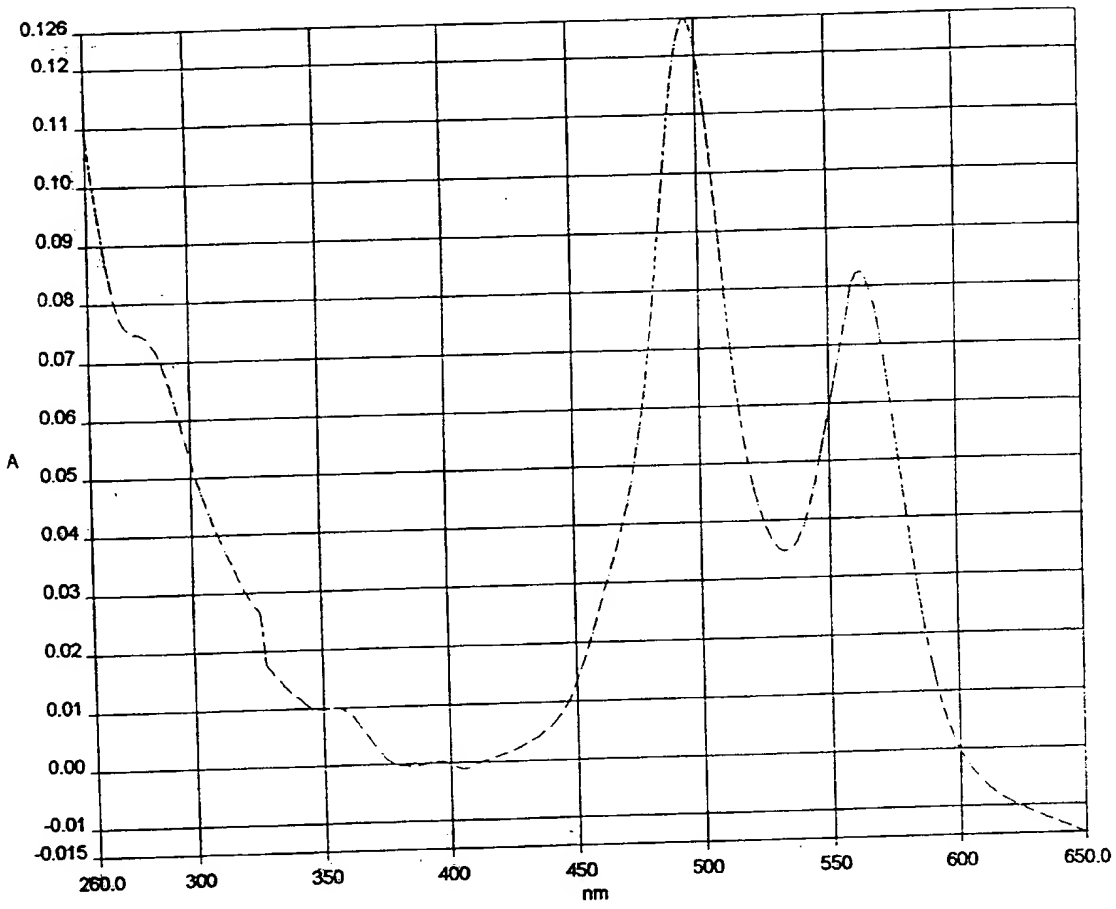
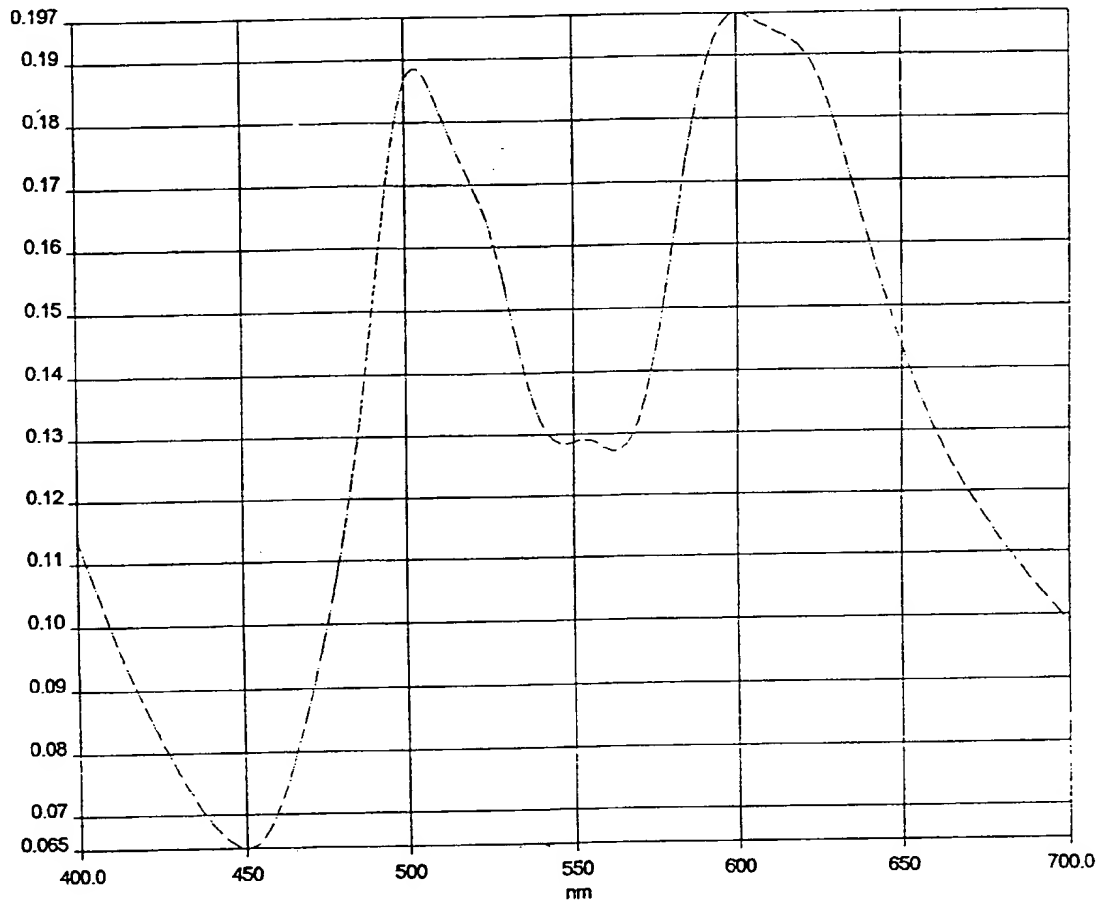


Fig. 12



FAMTAMRA-Lys$_6$-11-ddATP

Fig. 13



FAMROX-Lys$_6$-11-ddATP

Fig. 14

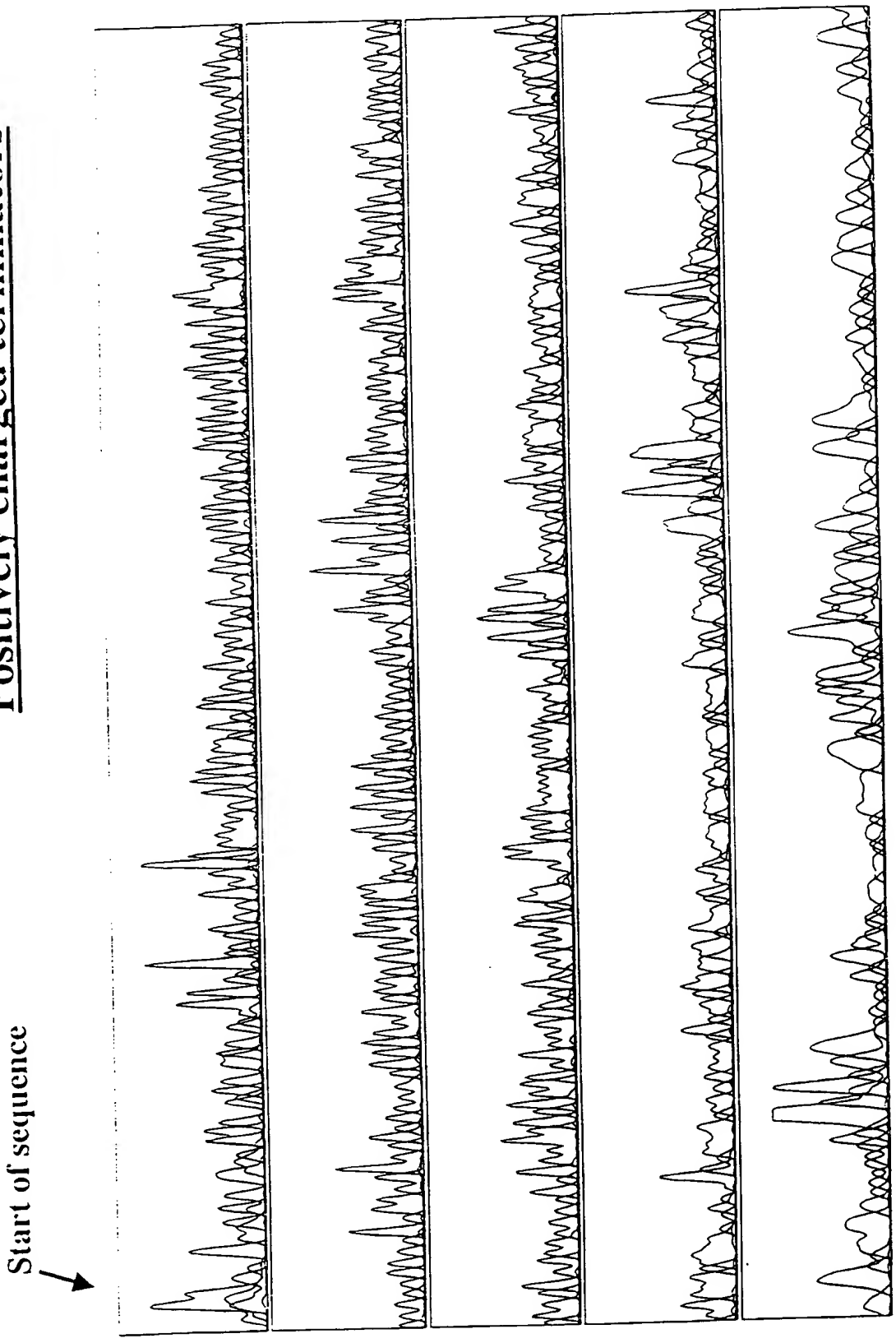


Fig. 15

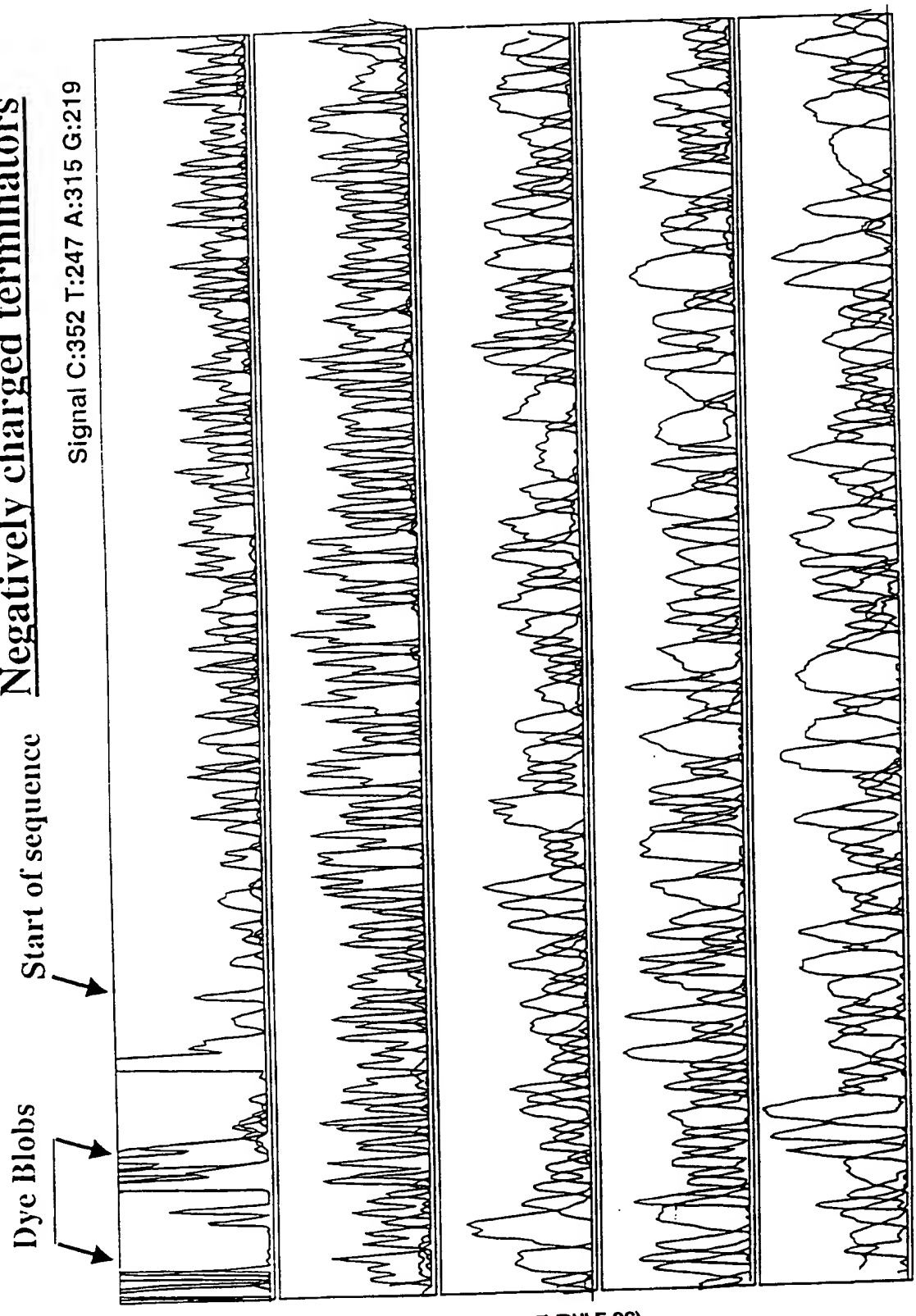

Negatively charged terminators
Signal C:352 T:247 A:315 G:219
Start of sequence
Dye Blobs

Fig. 16

Slab gel, -3 charge ET terminators (pick #2), directly loaded

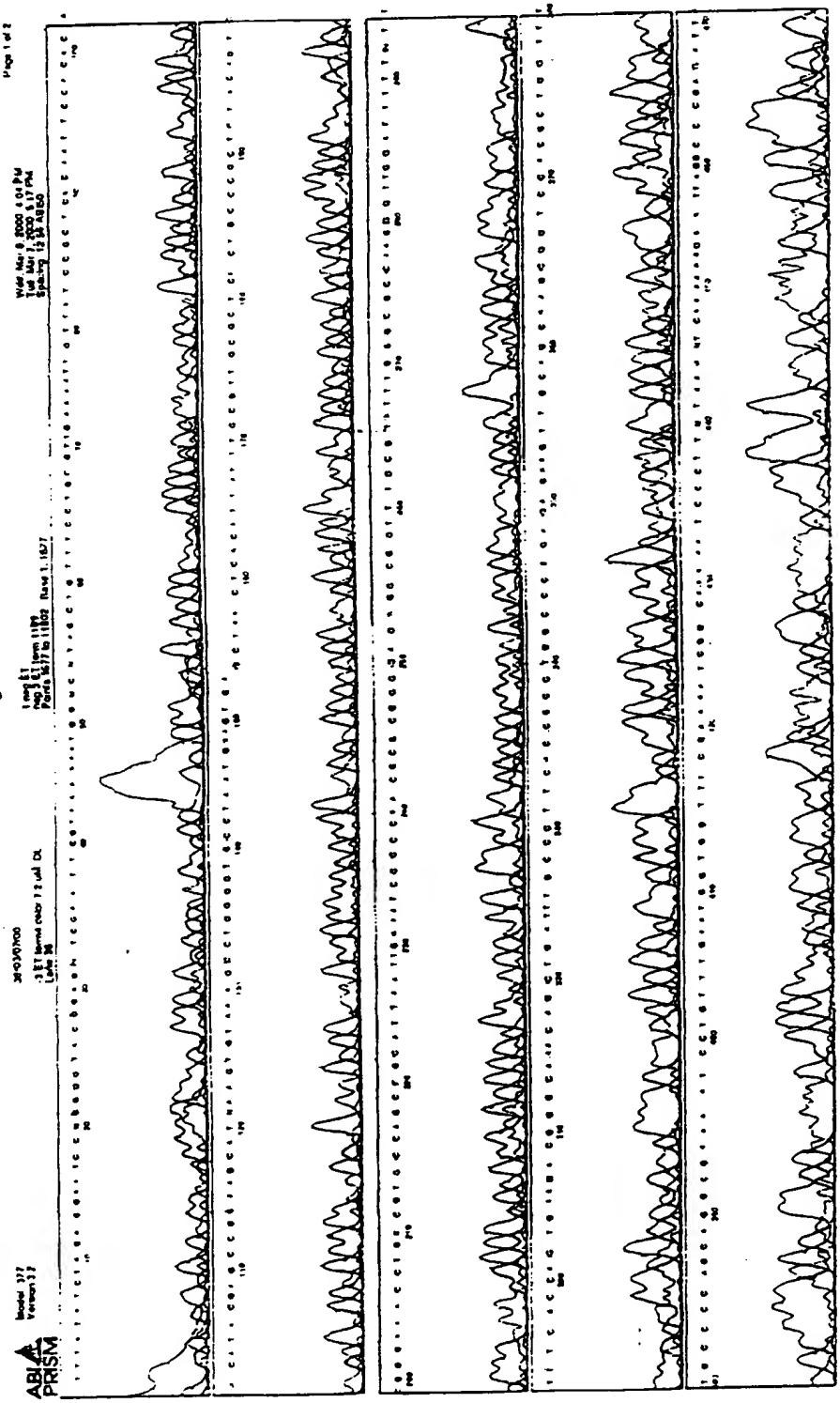


Fig. 17

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 00/25433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H19/10 C07H19/20 C12Q1/68 G01N33/53 A61K31/7052
A61P31/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07H C12Q G01N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 40223 A (AMERSHAM PHARM BIOTECH INC) 12 August 1999 (1999-08-12) the whole document ---	1,4,5, 7-9,12, 17-19
X	WO 98 58942 A (AMERSHAM PHARM BIOTECH INC) 30 December 1998 (1998-12-30) the whole document ---	1,4,5, 7-9,12, 17-19
X	WO 99 37810 A (AMERSHAM PHARM BIOTECH INC) 29 July 1999 (1999-07-29) the whole document ---	1,4,5,7, 9,12, 17-19
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 2001	22/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	de Nooy, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	T. YAMAGUCHI AND M. SANEYOSHI: "Synthetic nucleosides and nucleotides. XXXIV. Photoaffinity labeling of HIV reverse transcriptase: synthesis and utilization of 2',3'-dideoxy uridylate analogs bearing aryl(trifluoromethyl)-diazirine moiety" NUCLEOSIDES & NUCLEOTIDES, vol. 15, 1996, pages 607-618, XP002159716 page 607 -page 608 ---	1,4,7,9, 12,20
X	K. STOLZE ET AL.: "Synthesis of 3'-sugar- and base-modified nucleotides and their application as potent chain terminators in DNA sequencing" HELVETICA CHIMICA ACTA, vol. 82, 1999, pages 1311-1323, XP002159717 the whole document ---	1,4,5,7, 9,12,17
X	US 5 187 085 A (LEE LINDA G) 16 February 1993 (1993-02-16) abstract formulae 6-10 ---	1,4,7,9, 12,17-19
X	US 5 558 991 A (TRAINOR GEORGE L) 24 September 1996 (1996-09-24) abstract column 25 -column 26 ---	1,4,7,9, 12,17-19
X	WO 96 11937 A (STRATAGENE INC) 25 April 1996 (1996-04-25) page 14, structures 4 and 5 ---	1,4,7,9, 12
X	EP 0 731 178 A (HAMAMATSU PHOTONICS KK) 11 September 1996 (1996-09-11) example 1 (figure 7) ---	1,4,7,9, 12
X	P.N. CONFALONE: "The use of heterocyclic chemistry in the synthesis of natural and unnatural products" J. HETEROCYCLIC CHEM., vol. 27, 1990, pages 31-46, XP002159718 page 42-45 ---	1,4,5,7, 9,12
P,X	WO 00 13026 A (AMERSHAM PHARM BIOTECH INC) 9 March 2000 (2000-03-09) abstract --- -/--	1,4,7-9, 12,17-19

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INTERNATIONAL SEARCH REPORT

Inte: onal Application No
PCT/US 00/25433

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	T. SCHOETZAU ET AL.: "Synthesis of a fluorescent derivative of 6-N-'N-(6-aminohexyl)-carbamoyl)-2',3'-did eoxyadenosine 5'-triphosphate for detection of nucleic acids" J. CHEM. SOC., PERKIN TRANS. 1, 2000, pages 1411-1415, XP002159719 the whole document	1,4,5,7, 9,12, 17-19

4

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INTERNATIONAL SEARCH REPORT

information on patent family members

Inter ›nal Application No
PCT/US 00/25433

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9940223 A	12-08-1999	AU 2571799 A	23-08-1999
		EP 1060264 A	20-12-2000
WO 9858942 A	30-12-1998	US 5986086 A	16-11-1999
		EP 0989990 A	05-04-2000
WO 9937810 A	29-07-1999	EP 1071812 A	31-01-2001
US 5187085 A	16-02-1993	AT 191511 T	15-04-2000
		DE 69132097 D	11-05-2000
		DE 69132097 T	12-10-2000
		EP 0550646 A	14-07-1993
		JP 6061280 B	17-08-1994
		WO 9206219 A	16-04-1992
US 5558991 A	24-09-1996	US 5332666 A	26-07-1994
		AT 117316 T	15-02-1995
		DE 3750996 D	02-03-1995
		DE 3750996 T	13-07-1995
		DK 337687 A	03-01-1988
		EP 0252683 A	13-01-1988
		IE 66903 B	07-02-1996
		JP 2610782 B	14-05-1997
		JP 7005170 A	10-01-1995
		JP 1180455 A	18-07-1989
		JP 2088405 C	02-09-1996
		JP 7121239 B	25-12-1995
		JP 2781378 B	30-07-1998
		JP 9124636 A	13-05-1997
		JP 10158530 A	16-06-1998
		KR 9102227 B	08-04-1991
		NO 174369 B	10-01-1994
		PT 85238 A,B	01-08-1987
		US 5625081 A	29-04-1997
		US 5608063 A	04-03-1997
		US 5242796 A	07-09-1993
		US 5306618 A	26-04-1994
		AT 114651 T	15-12-1994
		CA 1340022 A	01-09-1998
		DE 3750792 D	12-01-1995
		DE 3750792 T	04-05-1995
		DK 81993 A	07-07-1993
		DK 82093 A	07-07-1993
		DK 337587 A	03-01-1988
		EP 0251786 A	07-01-1988
		ES 2066760 T	16-03-1995
		GR 3015197 T	31-05-1995
		IE 68058 B	15-05-1996
		JP 2097744 C	02-10-1996
		JP 8005908 B	24-01-1996
		JP 63152364 A	24-06-1988
		KR 9601528 B	01-02-1996
		KR 9605720 B	01-05-1996
		NO 872757 A,B,	04-01-1988
		PT 85237 A,B	01-08-1987
		US 5047519 A	10-09-1991
		US 5151507 A	29-09-1992

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter onal Application No
PCT/US 00/25433

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9611937 A	25-04-1996	AT 191917 T	15-05-2000
		CA 2202480 A	25-04-1996
		DE 69516415 D	25-05-2000
		DE 69516415 T	14-09-2000
		EP 0785941 A	30-07-1997
		JP 10509429 T	14-09-1998
EP 0731178 A	11-09-1996	JP 8313529 A	29-11-1996
		US 5843658 A	01-12-1998
WO 0013026 A	09-03-2000	AU 5697099 A	21-03-2000
		DE 29915165 U	02-03-2000
		GB 2341189 A	08-03-2000